Histologic and Genetic Characterisation of HIV-1 Infecting the Lymphoid and non-Lymphoid Tissues in Different Stages of Infection

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2000

獻給我最親愛的

祖母，爸爸，媽媽，弟弟，妹妹

婷慧· 愛丁堡

Declaration

I declare that all the work presented in this thesis is of my own composition, and the studies described was undertaken by myself.

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Abbreviations

3TC	Lamivudine
Ab	Antibody
ABC	Avidin-Biotin complex
ADCC	Antibody dependent cell mediated cell cytotoxicity
ADM	AIDS drug misuser
Ag	Antigen
AIDS	Acquire immunodeficiency syndrome
AP	Alkaline-phosphotase
APCs	Antigen presenting cells
ARC	AIDS related complex
ARD	AIDS related dementia
ARV	AIDS-associated retroviruses
ATP	Adenosine tri-phosphate
AZT	Zidovudine
BBB	Brain blood barrier
BMVECs	Brain microvascular endothelial cells
BSA	Bovine serum albumin
CA	Capsid antigen
CARD	Catalysed reporter deposition
CD	Cluster determinant
CDC	Centers for Disease Control
cDNA	Complement DNA
CDR	Complementarity determining region
CMV	Cytomegalovirus
CNS	Central nervous system
cPPT	Central polypurine track
CTLs	Cytotoxic T lymphocytes
CTS	Central termination signal
d4T	Stavudine
DAB	Diaminobenzidine
DC	Dendritic cell
ddC	zalcitabine
ddI	Didanosine

DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulphoxide
DNA	Dideoxyribonucleic acid
DNase	Dideoxyribonuclease
dNTPs	Dinucleotide tri-phosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassays
ELISA	Enzyme-linked immunosorbent assay
Env	The virus envelope
Env gp	Envelope glycoprotein
ER	Endoplasmic reticulum
Fc	Fragment crystalline
FDC	Follicular dendritic cell
GC	Germinal centres
GFAP	Glial Fibrillary Acidic Protein
GI	Gastrointestinal system
GM-CSF	Granulocyte-macrophage colony stimulating factor
Gp120	External (or surface) envelope glycoprotein
Gp41	Transmembrane envelope glycoprotein
HAART	Highly active antiretroviral therapy
HEPA	High efficiency particulate absorption
HIV	Human immunodeficiency virus
HIVE	HIV-encephalitis
HLA	Human leucocyte group A
HRP	Horseradish-peroxidase
HTLV	Human T lymphotropic virus
ICTV	International Committee on Toxonomy of Viruses
IDU	Intravenous drug use
IF	Immunofluorescence
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL-2	Interleukin 2
IMS	Industrial methylated spirits

IN	Integrase
Kb	Kilo-base
KDa	Kilo-Daltons
KS	Kaposi's sarcoma
LAS	Lymphadenopathy syndrome
LAV	Lymphadenopathy associated virus
LLP	Lentivirus lytic peptides
LTR	Long terminal repeat
MA	Matrix protein
mAb	Monoclonal antibody
MAI	*Mycobacteria avium-intracellulare*
MGCs	Multinucleated giant cells
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MIS	Mucosal immune system
MRC	Medical Research Council
mRNA	Messenger RNA
NC	Nucleocapsid protein
Nef	Negative Factor
NK	Natural killer cell
NLS	Nuclear localisation signal
NMDA	N-methyl-D-aspartate
NSI	Non-syncytium inducing
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCP	*Pneumocystis carinii* Pneumonia
PCR	Polymerase chain reaction
PGL	Persistent generalised lymphadenopathy
PIC	Pre-integration complex
PML	Progressive multifocal leukoencephalopathy
PND	Principal neutralising domain
PPT	Polypurine track
PR	Protease
R	Repeat region
RANTES	Regulated-upon-activation normal T expressed

Rev	Regulator of viral expression
RNA	Ribonucleic acid
RNase	Ribonuclease
RRE	Rev responsive element
RT	Reverse transcriptase
RTC	Regional transfusion centre
SA	Streptavidin
SDS	Sodium-*n*-lauroylsarcosine
SI	Syncytium inducing
SIV	Simian immunodeficiency virus
snRNPs	Small ribonuclear proteins
Taq	Termus aquaticus
TAR	Tat response element
Tat	Transcriptional transactivator
TCR	T cell receptor
TNF	Tumour necrosis factor
tRNA	Transfer RNA
TSA	Tyramide signal amplification
UNAIDS	Joint United Nations Programme on HIV/AIDS
VCAM-1	Vascular cell adhesion molecule 1
Vif	Viral infectivity factor
VIP	Vacuum Infiltration Processor
Vpr	Viral protein R
Vpu	Viral protein U
Vpx	Viral protein X

Abstract

The HIV epidemic continues to spread at a rate of over 6000 new infections per day. A better understanding of HIV pathogenesis and the mechanisms underlying HIV-1 related disease in specific tissues such as the brain is still a high priority in tracking the medical impact of the pandemic. This project was aimed to investigate the cellular localisation of HIV-1, cytopathology and viral phenotype variation in various organs throughout the course of HIV-1 infection. The cellular localisation of HIV-1 infection was demonstrated by immunohistochemistry, and the virus distribution, genotypes and inferred phenotype and virion diversity were determined by nucleotide sequencing.

Immunohistochemistry applied in serial tissue sections or combining the double-labelling technique with various cell markers was employed to examine the variety of HIV-1 infected cell populations. During the clinically latent stage, HIV-1 p24 signal was detected primarily within lymphoid organs and occasionally within lung tissue. However, in the symptomatic stage, the p24 signal was detected within a wider range of organs, including lymph node, spleen, lung, ileum, colon, liver, kidney and central nervous system. Among the lymphoid organs, p24 antigen was localised to follicle centres and intimately colocalised with CD21-positive follicular dendritic cells. In lung and intestine, detection of p24 antigen was confined to secondary lymphoid tissue, primarily associated with CD21-positive cells and occasionally with tissue macrophages and T-cells. In brain tissue, the HIV-1 p24 positive cells were identified as microglia and were a defining feature of HIV-encephalitis.

The distribution of the HIV-1 co-receptors CXCR4 and CCR5 was also examined by immunohistochemical techniques in these organs. The staining patterns of CXCR4 and CCR5 positivity showed overlap. Commonly, CXCR4 and CCR5 expression was detected on neurones, epithelium cells, blood vessel endothelium cells, tissue macrophages and occasionally lymphocytes. Interestingly, in the lymphoid aggregates or lymphoid follicles where p24 antigen was generally detected, neither CXCR4 nor CCR5 expression was detected.

The *in vitro* phenotype was predicted according to the amino acid sequences in V3 region obtained from direct isolates. Most of the variants isolated from various organs were CCR5-dependent (non-syncytium inducing) strains. CXCR4-dependent (syncytium inducing) variants were found preferentially in individuals with low CD4-positive cell counts at death, and in the male homosexual risk group. Phylogenetic analysis of nucleotide sequences from the V3 and $p17^{gag}$ regions revealed a variety of relationships between variants recovered from different tissues. HIV-1 proviral DNA was detected in all lymph node and lung samples. Variants from each tissue were invariably interspersed suggesting a shared population between these tissues and target cell type. The detection of proviral DNA in brain was strongly associated with HIV-encephalitis. Variants detected in the brain had a predicted CCR5-dependent phenotype and were phylogenetically distinct from the variants from lymph nodes. Sequence relationships between variants in the colon were more complex. Colon variants from two study subjects showed sequences interspersed with lymph node and lung isolates. However variants from the colon of three other individuals were genetically distinct from those in lung and lymph node, and more similar to those found in the brain.

Discordant phylogenetic relationships between $p17^{gag}$ and V3 regions of HIV-1 genome were observed in some samples. For example, separate grouping of $p17^{gag}$ region sequences from lymph node and colon were observed in two study subjects while sequences in V3 from these tissues were interspersed. Similarly in another individual, $p17^{gag}$ sequences from the brain were extremely diverse and polyphyletic, while V3 sequences clustered into two discrete clades. These observations suggest genetic recombination between different evolutionary lineages of HIV.

Together these results suggested that there may be at least two mechanisms of spread of HIV-1 into non-lymphoid tissues. For tissues such as the lung and the gastrointestinal tract, combined immunohistochemical and genetic observations suggest that infection of these tissues arose directly from infiltration by lymphocytes and other lymphoid cells. A different mechanism seems likely for HIV-1 variants detected in the brain, which were found predominantly in microglial cells, and these

formed genetically distinct populations from those in lymphoid tissues. In three of the study subjects, in whom HIV-1 proviral sequences were detected in the colon, there was also evidence for tissue specificity, consistent with infection of cells distinct from those in lymphoid tissue, although p24 immunostaining failed to identify the infected cell type.

In the future, *in-situ* PCR for HIV proviral sequences combined with immunostaining for cell surface markers might be employed to investigate the cell types which are latently or non-productively infected and therefore provide a more complete description of the cellular targets of HIV-1 *in vivo*. Phenotypic characterisation of HIV-1 variants infecting different cell types would also be of value in understanding the mechanism of the observed genetic compartmentalisation between tissues, and the extent to which sequence differences represent adaptation for replication in different cell types.

Chapter 1: General Introduction

1.1 Background

1.1.1 Acquired Immunodeficiency Syndrome

Acquired immunodeficiency syndrome (AIDS), which was until very recently an almost inevitably fatal disease in the modern world, is caused by infection with the human immunodeficiency virus (HIV), and is characterised by a long latent period prior to the development of symptomatic diseases.

The disease first came to public notice in the summer of 1981. A rare type of pneumonia, *Pneumocystis carinii* pneumonia (PCP), was reported by the U.S. Centers for Disease Control (CDC) in previously healthy gay men (Gottlieb *et al.*, 1981; CDC, 1981). Later that year, an unusual vascular cancer, Kaposi's sarcoma (KS), and other opportunistic infections were also reported in previously healthy homosexual groups (Durack, 1981; Thomsen *et al.*, 1981; Friedmankien *et al.*, 1982; Gottlieb & Ackerman, 1982). This was taken as signaling a new acquired cellular immunodeficiency since PCP and KS were varieties of opportunistic infection commonly associated with immune-compromised patients who received immunosuppressive drug therapy, but now affecting homosexuals who did not display an obvious reason for being immunosuppressed. Subsequently, faced with a rapidly growing number of cases not only among homosexuals, but also in intravenous drug users and haemophiliacs (CDC, 1982), U.S. health officials utilised the term acquired immunodeficiency syndrome (AIDS) for this new disease.

1.1.2 Discovery of The AIDS Virus

Clinical findings suggested that the course of the disease was characteristically that of an immune disorder, developing during a long term latent period, and finally ending with severe illnesses such as opportunistic infections, neurological disorders, and neoplastic complications. Epidemiological studies suggested that the infectious agent of AIDS could be transmitted during sexual intercourse, through intravenous

drug abuse, or by therapies utilising contaminated blood and blood products (CDC, 1982; Marmor *et al.*, 1982; Jaffe *et al.*, 1983; Cowan *et al.*, 1984). Because infection with the prototype members of the lentivirus genus are commonly associated with a variety of neurological and immunological diseases such as autoimmunity, pneumonitis, brain and joint disorders (Haase, 1986; Coffin, 1996; Levy, 1998), as AIDS researchers looked for a pathogen which would first cause an immune disorder in humans, and later developed neurological, neoplastic complications and opportunistic infections, a human lentivirus was an obvious suspect.

In 1983, a French group which led by Dr. Barré-Sinoussi in the Pasteur Institute recovered a virus containing reverse transcriptase (RT) from an enlarged lymph node of a gay man with persistent multiple lymphadenopathy syndrome (LAS) (Barré-Sinoussi *et al.*, 1983). This was the first evidence that AIDS might be caused by a retrovirus, since the virally encoded RT is known to be the major character of retroviruses, and the LAS was confirmed as an AIDS-related syndrome (Barré-Sinoussi *et al.*, 1983). This virus was similar to the human T-lymphotropic virus (HTLV) in infecting CD4+ lymphocytes. However, it grew to substantial titer in CD4+ lymphocytes and killed them, instead of establishing the cells in culture as does HTLV (Montagnier *et al.*, 1984). This virus was later named lymphadenopathy-associated virus (LAV) by Montagnier and coworkers (Montagnier *et al.*, 1984).

Later, Gallo and associates isolated a virus from the peripheral blood mononuclear cells (PBMCs) of adult and paediatric AIDS patients (Gallo *et al.*, 1983; Gallo *et al.*, 1984; Popovic *et al.*, 1984). This virus was lymphotropic, and reported to cross-react with some viral proteins of HTLV-I and HTLV-II, particularly the core p24 protein (Gallo *et al.*, 1984). Thus, this virus was named HTLV-III. They believed that it merited inclusion in the HTVL group, even though the newly isolated virus was cytolyic and did not induce an established cell line from infected lymphocytes.

At the same time, Levy and coworkers also recovered viruses from AIDS patients from different known risk groups (Levy *et al.*, 1984a; Levy *et al.*, 1984b; Levy *et al.*, 1985). Their viruses, which were named AIDS-associated retroviruses (ARV) showed some cross-reactivity with the French LAV strain. It was also observed that ARV grew substantially in PBMCs, killed CD4+ lymphocytes, and did not immortalise them (Levy *et al.*, 1984a).

Subsequently, the three prototype viruses, LAV, HTLV-III and ARV were recognised as members of the same group of retroviruses, and their properties identified them as members of the lentivirus genus of the family *Retroviridae*. These AIDS viruses had many properties distinguishing the from HTLV, in 1986 the International Committee on Taxonomy of Viruses (ICTV) recommended giving the AIDS viruses a separate name, human immunodeficiency virus (HIV) (Coffin *et al.*, 1986). Soon after the recognisation of HIV, a unique lentivirus discovered by Clavel and coworkers has also been linked to AIDS in West Africa (Clavel *et al.*, 1986; Clavel *et al.*, 1987). Therefore, HIV-1 refers to the genetically related viruses found in several regions of Africa, Asia, Europe and both North and South America, and HIV-2 is the distinct virus prevalent in certain West African countries (Hirsch & Curran, 1996; Luciw, 1996).

1.1.3 Classification of Human Immunodeficiency Viruses

It has been confirmed that HIV is a member of *Lentivirina*, one of the seven genera in the *Retroviridae* family of RNA viruses.

Retroviridae was named because they possess reverse transcriptase (RT). Based on their pathogenicity, the *Retroviridae* family were originally classified into three subfamilies: *Oncovirinae*, *Lentivirinae*, and *Spumavirinae*. Oncoviruses are the only genus of retroviruses that can transform target cells, and are associated with cancers and neurological disease. Lentiviruses are responsible for a variety of neurological and immunological diseases, but are not directly implicated in any malignancies. Spumaviruses appear to cause a vacuolated "foamy" cytopathological effect but not

effect but not clinical disease (reviewed in Coffin, 1996). Subsequently, in the late 1980s, as nucleotide sequence relationship and genome structure were introduced as criteria for virus classification, the ITCV then redefined the taxonomy of the *Retrovirida*. According to virion structure, utilisation of particular cell receptors, lifestyle, presence or absence of an oncogene, and other pathogenic properties, viruses were classified into seven distinct genera, including Avian-leukosis-sarcoma, Mammalian C-type, B-type viruses, D-type viruses, HTLV-BLV group, Spumavirus group and Lentivirus group (Figure 1. 1) (reviewed in Coffin, 1996).

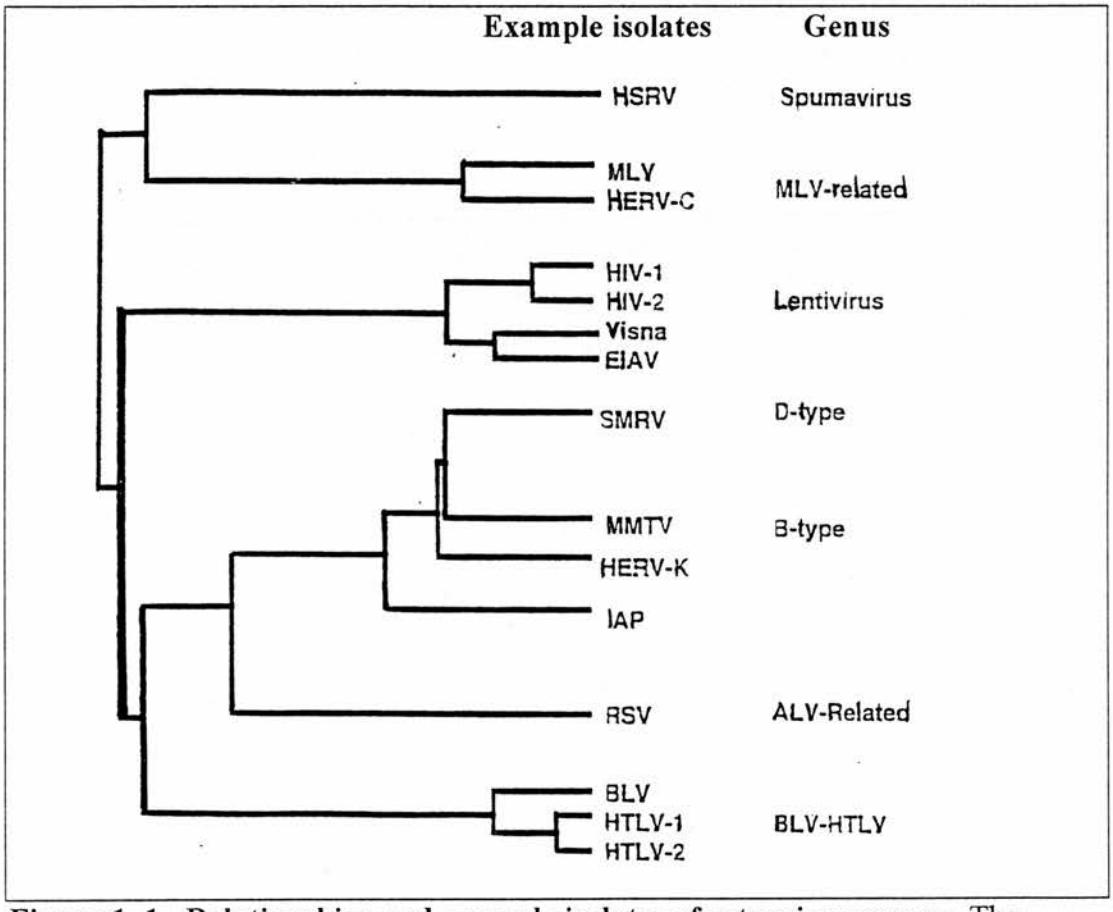


Figure 1. 1. Relationships and example isolates of retrovirus groups. The relationships are based on amino acid sequence similarities in the RT protein of the group shown. **HSRV:** Human spumaretrovirus; **MLV:** Murine leukemia viruses; **HERV-C:** Human endogenous retrovirus type C; **HIV-1& HIV-2:** Human immunodeficiency virus type 1 and type 2; **Visna:** Visna.maedi virus; **EIAV:** Equine infectious anemia virus; **SMRV:** Squirrel monkey retrovirus; **MMTV:** Mouse mammary tumor virus; **IAP:** intracisternal A particle; **RSV:** Rous sarcoma virus; **BLV**: Bovine leukemia virus; **HTLV-1 & HTLV-2:** Human lymphotropic virus type 1 and 2. (Modified from *Fields Virology*, 1996).

Lentiviruse genus includes complex exogenous viruses responsible for a variety of neurological and immunological diseases. The prototype members of this family were the "slow" viruses visna, equine infectious anemia virus, and caprine arthritis-encephalitis virus. More recent isolates include HIV and simian immunodeficiency viruses (SIV), and the more distantly related feline and bovine immunodeficiency viruses (FIV and BIV). Characters and phylogenetic relationships of these viruses are described in Table 1. 1 & Figure 1. 2.

Table 1. 1. Clinical disorder and host range of lentiviruses.

(Modified from *HIV and the Pathogenesis of AIDS*, Levy, 1998)

Virus	Host Infected	Clinical disorder
Equine infectious anaemia virus	Horse	Cyclical infection in the first year; Autoimmune; Haemolytic anaemia; sometimes Encephalopathy
Visna / maedi virus	Sheep	Encephalopathy / Pneumonia
Caprine arthritis-encephalitis virus	Goat	Immune deficiency; Arthritis; Encephalopathy
Bovine immune deficiency virus	Cow	Lymphadenopathy; Lymphocytosis; Central nervous system disease
Feline immunodeficiency virus	Cat	Immune deficiency
Simian immunodeficiency virus	Primate	Immune deficiency and Encephalopathy
Human immunodeficiency virus	Human	Immune deficiency and Encephalopathy

According to the distinctly serologic properties, and the differences observed in sequence analysis, HIV has been grouped into two types, HIV-1 and HIV-2 (Clavel *et al.*, 1986; Coffin *et al.*, 1986). Both HIV-1 and HIV-2 are a cause of AIDS, and the modes of transmission are similar (Markovitz, 1993). However, some investigators have reported that the transmissibility and pathogenic potential of HIV-2 strains are lower than HIV-1 strains. It has been found also, individuals infected with HIV-2 appears to have a longer pre-symptomatic period and a lower cytopathogenicity than those infected with HIV-1 (Hirsch & Curran, 1996; Levy, 1998).

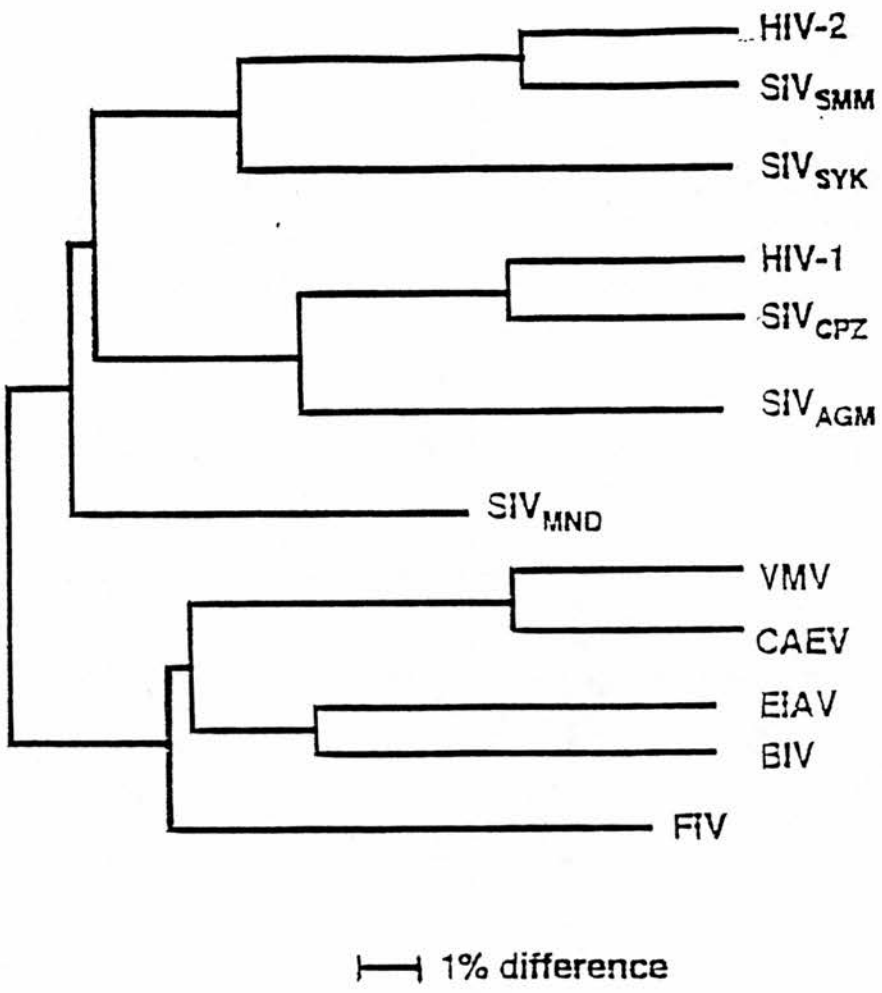


Figure 1. 2. Phylogenetic relationships of lentiviruses. Representative lentiviruses are compared using *pol* gene nucleotide sequences for establishing phylogenetic relationships. Five groups of primate lentiviruses are shown: **HIV-1**, **HIV-2**, **SIV** from sooty mangabey monkey (**SIV**$_{SMM}$), Sykes monkey (**SIV**$_{SYK}$), chimpanzee (**SIV**$_{CPZ}$), African green monkey (**SIV**$_{AGM}$) and mandrill (**SIV**$_{MND}$). Nonprimate lentiviruses are Visna / maedi virus (VMV), Caprine arthritis-encephalitis virus (**CAEV**), Equine infectious anaemia virus (**EIAV**), Bovine immune deficiency virus (**BIV**), and Feline immunodeficiency virus (**FIV**). The scale indicates percentage difference in nucleotide sequences in the *pol* gene. (Copied from *Fields Virology*, 1996.)

HIV-2 was first recovered in Portugal from AIDS patients from West Africa (Clavel *et al.*, 1986). After cloning and sequence analysis, it was found that this virus differed by more than 55% from the HIV-1 strains, and appeared to be antigenically distinct (Clavel *et al.*, 1986). Also, the genome of HIV-2 presents a unique accessory gene, *vpx*, which does not exist in HIV-1 genome. In contrast, the genome of HIV-1 presents the *vpu*, which is absent in HIV-2 genome (Figure 1. 3). The major serologic difference between HIV-1 and HIV-2 resides in the envelope glycoproteins. Using immunoblot analyses, antibodies to HIV-2 would generally cross-react with the Gag and Pol proteins of HIV-1, but might not detect HIV-1 envelope proteins and vice versa (Clavel *et al.*, 1987; Levy, 1998). In fact, the envelope glycoproteins of HIV-2 appear to cross-react serologically with envelope proteins from strains of simian immunodeficiency virus (SIV), a complex group of primate lentiviruses. Most commonly with wild sooty mangabeys SIV (SIV_{sm}) (Clavel *et al.*, 1987; Chen *et al.*, 1997).

Both HIV-1 and HIV-2 could further divided into several groups. According to the extraordinary sequence diversity in the *env* gene, HIV-1 is currently divided into three groups, M, O and N. "M" accounting for the major group, which comprises ten worldwide distributed genetically distinct subtypes (A through J). "O" refers to the outlier group, which consists of a pool of highly divergent, genetically related strains with no defined clade. The distribution of the "O" group is localised in Cameroon, Gabon, and Equatorial Guina (Quinn, 1996). Group N is recently identified in 1998 from three people in Cameroon by French and African scientists (Simon *et al.*, 1998). This highly divergent HIV-1 isolate is phylogenetically situated between group M of HIV-1 and chimpanzee simian immunodeficiency virus (SIV_{cpz}), but far from group O. The letter "N" is then designated to signify this divergent strain as "non M-non O" group. As for HIV-2, at least five subtypes (A through E) are identified, and most of the HIV-2 strains are localised in Africa, especially in the western regions (Quinn, 1996).

The focus of studies described in this thesis is HIV-1. Therefore, HIV-2 will not be described further.

1.2 The HIV-1 Virion

1.2.1 Genomic Organisation

HIV-1 is an RNA virus, which belongs to the genus of lentiviruses of the *Retroviridae* family. The size of the HIV-1 genome is about 9-10 kb, which is bounded by the non-coding long terminal repeat regions (LTR) at both 3' and 5' end, with several open reading frames coding for viral proteins. Similar with other lentiviruses, the HIV-1 genome contains three principal structural genes, *gag*, *pol* and *env,* which encode essential constructing precursor proteins for the virion (Figure 1. 3).

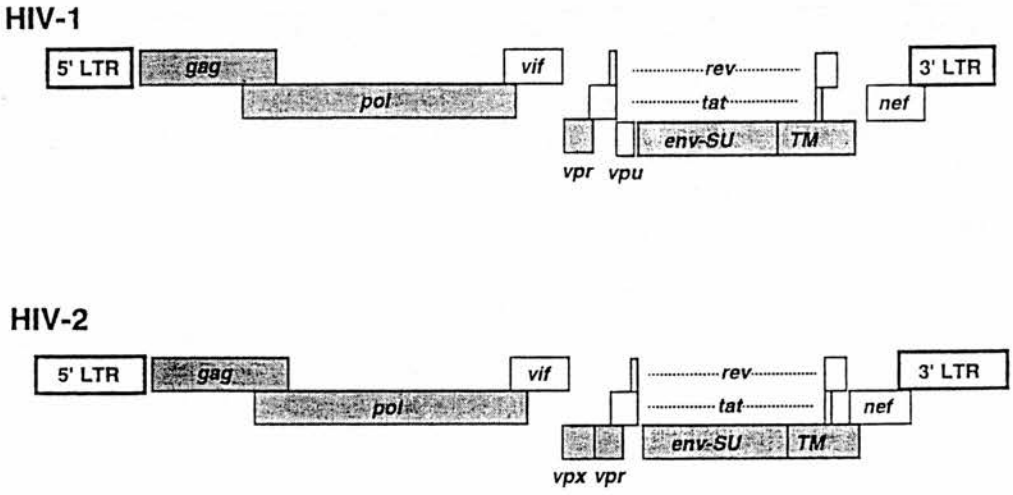


Figure 1. 3. Genome organisation of HIV-1 and HIV-2. In general, the genome of HIV-1 and HIV-2 is known to have nine genes, including three principal structural genes (*gag*, *pol* and *env*), and six accessory genes (*vif*, *vpr*, *rev*, *tat*, *nef* and *vpu* (HIV-1) or *vpx* (HIV-2)), which is bounded by the LTR at both 3' and 5' end. The relative positions of the known accessory genes (not drawn to scale) are shown in relationship to the *gag, pol* and *env* genes. Note the unique presence of *vpu* in HIV-1 and *vpx* in HIV-2. (Modified from *Fields Virology*, 1996).

The *gag* gene (group-specific antigens) is located immediately at downstream of 5'-LTR gene, and encodes a 55 kDa precursor protein ($Pr55^{gag}$). This gag precursor protein is then proteolytically cleaved into smaller proteins, including Gag p24 capsid protein (CA), matrix p17 protein (MA), nucleocapsid p9 and p7 proteins (NC). Located between *gag* and *env* genes, HIV-1 *pol* gene encodes three virus enzymes, reverse transcriptase, integrase, and protease. Initially, all these *gag* and *pol* products are presented as a 160 kDa Gag-Pol precursor protein ($Pr160^{gag\text{-}pol}$). The viral protease is then employed to cleave $Pr160^{pol\text{-}gag}$ and $Pr55^{gag}$ into individual proteins. The *env* gene encodes envelope 160 kDa precursor glycoprotein, gp160. This precursor gp160 is then cleaved by cellular protease to generate an external envelope glycoprotein, gp120, and an anchoring transmembrane glycoprotein, gp41 (reviewed in Luciw, 1996).

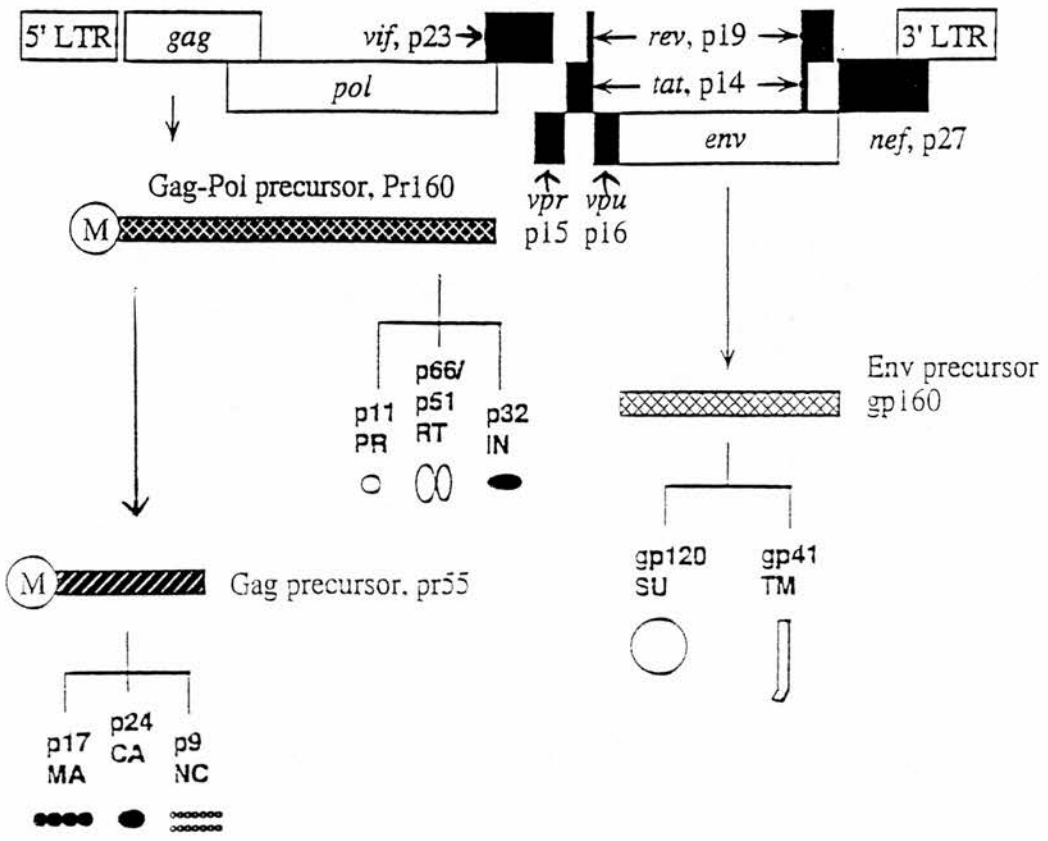
Apart from these essential structural genes, the HIV-1 genome also contains six regulatory genes, including *tat*, *rev*, *vif, vpu, vpr*, and *nef. Tat* and *rev* encode small non-structural viral proteins, which are required for viral replication. *Vif, vpu, vpr,* and *nef* belong to non-essential genes, also named as "accessory" or "auxiliary" genes, which work as the regulators for virus replication.

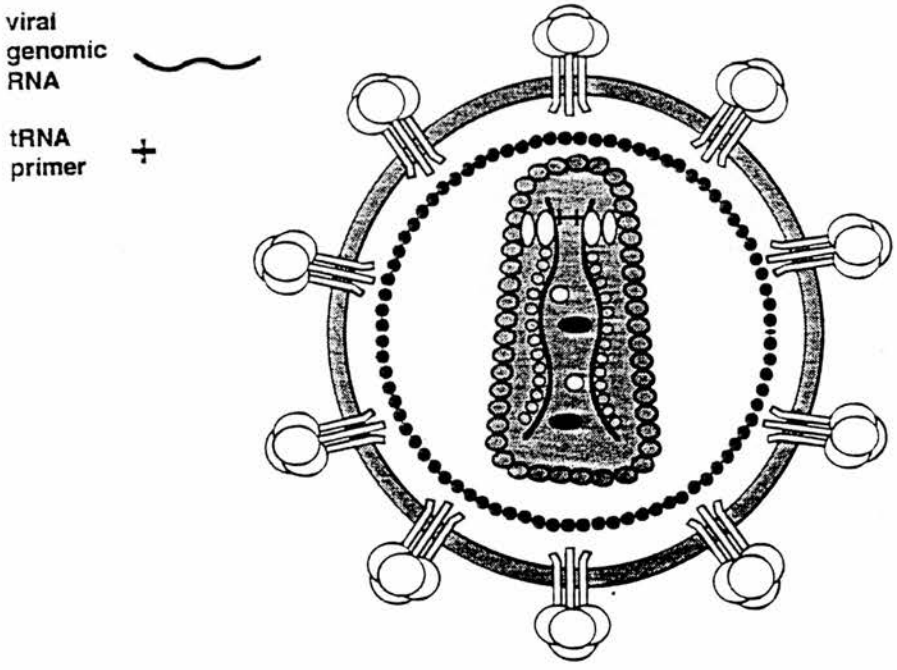
1.2.2 Virion Structure

The HIV-1 virion has a spherical shape, and is about 110 nm in diameter. It consists of a lipid bilayer envelope, which surrounds the cone-shaped virus capsid. The cone-shaped virus capsid is about 100 nm in the long dimension with one 40 to 60 nm wide end, and another 20 nm narrow end. It is the main part of the HIV-1 virion and composed of the viral Gag p24 capsid protein (Gelderblom *et al.,* 1989; reviewed in Luciw, 1996). Inside this capsid, there are two identical single-stranded RNAs, with which the viral reverse transcriptase (p51 and p66), protease (p11), and integrase (p32) are closely associated with the nucleocapsid proteins (p7 and p9). Surrounding the viral capsid, there is a matrix protein, p17, which is located underneath the viral envelope.

Figure 1. 4. Processing of viral proteins and virion structure. The top portion of the figure shows the processing of HIV-1 viral proteins. Precursor polyproteins Gag-Pol ($Pr160^{gag\text{-}pol}$), Gag ($Pr55^{gag}$), and Env (gp160) are enzymatically processed to yield mature virion proteins. The $Pr160^{gag\text{-}pol}$ and $Pr55^{gag}$ are processed by the viral aspartic protease into several smaller proteins, which include matrix (MA, p17), capsid (CA, P24), Nucleic acid binding (NC, P9), proline-rich (P6), transcriptase (RT, p51 and p66), protease (PR, p11), and integrase (IN, p32). The Env gp160 is processed by a cellular protease into the surface glycoprotein (SU, gp120) and transmembrane protein (TM, gp41). Of the accessory proteins encoded by HIV-1, only Vpr is found to assemble into virions, but the precise location in virus particles has not been determined. Neither the other accessory proteins (Vif, Vpu and Nef) nor the regulatory proteins (Tat and Rev) have been detected in virion particles. Symbols representing the various virion proteins are indicated. (M: myristoylated).

(Modified from *Fields Virology*, 1996 and *HIV and the Pathogenesis of AIDS*, Levy, 1998).


5' LTR
gag
vif, p23
rev, p19
3' LTR
pol
tat, p14
env
nef, p27
vpr
vpu
p15
p16
Gag-Pol precursor, Pr160
M
p66/
p51
p11
PR
RT
p32
IN
Env precursor
gp160
gp120
SU
gp41
TM
M
Gag precursor, pr55
p17
MA
p24
CA
p9
NC


viral
genomic
RNA
tRNA
primer

On the surface of the lipid bilayer envelope, there stand approximately 72 spikes. Each spike is about 8 to 10nm long and contains three or four heterodimers. Each heterodimer consists of two envelope glycoproteins that present an ovoid distal end, and a stem, which links the ovoid end to the lipid envelope. The ovoid distal end is composed of the external surface envelope glycoprotein, gp120, and the stem is composed of the transmembrane envelope glycoprotein, gp41. These two subunits are non-covalently linked together (reviewed in Luciw, 1996). The schematic model of mature HIV-1 virion is shown in Figure 1. 4.

1.2.3 The Functions of Viral Proteins

1.2.3.1 Core Proteins

Matrix p17 (MA), capsid p24 (CA) and nucleocapsid p7 and p9 (NC) proteins are the main components of the HIV core region which are employed for virion morphogenesis. They function to package genomic viral RNA into the virion and are possibly involved in the early steps of the viral replication cycle (reviewed in Luciw, 1996).

Gag p24 Capsidprotein (CA)

Capsid protein is the major subunit of the virus capsid, which is released from the central portion of the $Pr55^{gag}$ by two cleavages mediated by viral protease (PR), and displays a high degree of hydrophobicity. The mature form of CA contains about 240 amino acids, and presents a molecular weight of 24 to 27 kDa (reviewed in Luciw, 1996). The actual functions of CA are not clear yet. Evidence from genetic studies suggests that one domain locating in the C-terminal portion of CA might govern nucleocapsid assembly (Dorfman *et al.*, 1994a). Other studies, based on *in vitro* systems, indicate that specific interactions of CA with the viral RNA genome and other Gag proteins may play a role during capsid assembly, however this needs further exploration (reviewed in Luciw, 1996).

NucleoCapsid protein (NC)

NC is a hydrophilic protein, which is cleaved from the C-terminus of $Pr55^{gag}$ by viral protease (PR), and links genomic viral RNA to the capsid shell. The mature form of NC protein contains about 70 amino acids, and shows a molecular weight of 7 to 9 kDa. Two copies of a special cysteine-histidine motif (Cys-X_2-Cys-X_4-His-Cys) are found incorporated in the NC protein. The second cysteine-histidine motif might be functionless; however, genetic studies with site-specific mutations in the viral genome have demonstrated that the first cysteine-histidine motif is important for nucleocapsid assembly. Further evidence from *in vitro* binding studies indicate that the first cysteine-histidine motif is also essential for the efficient and specific interaction with the major packing site on genomic viral RNA. This interaction is responsible for the formation and stability of the dimeric form of viral RNA in virions (reviewed in Luciw, 1996).

Matrix p17 protein (MA):

MA provides a matrix for the viral structure and is essential for the virion integrity. It is cleaved from the N-terminus of $Pr55^{gag}$ by viral protease, contains 130 amino acids, and its molecular weight is about 17 to 18 kDa. MA is required for the viral assembly (Hunter, 1994). Mutagenesis studies have demonstrated that deletions in the first two-thirds of HIV-1 MA will cause a failure in the incorporation of envelope glycoproteins into the mature virion, although the processing of the gag proteolysis or virion assembly and release might not be affected (Dorfman *et al.*, 1994b). In addition, noninfectious virions are produced when cultured cells are transfected with HIV-1 containing small deletion mutations at the C-terminus of MA. This result suggests that MA is also important for the early stages of viral infection (reviewed in Luciw, 1996).

1.2.3.2 Viral Enzymes

Protease (PR), integrase (IN), reverse transcriptase (RT) are the three viral enzymes of HIV-1, which are the products of *pol* gene. All of them are very important and indispensable for the replication of HIV-1.

Protease (PR)

Protease contains 99 amino acids and its molecular weight is about 10 kDa (Katz & Skalka, 1994). During post-translated processing of viral proteins, the mature HIV-1 protease dimer is first released from the $Pr160^{gag\text{-}pol}$ by an autocatalytic cleavage. HIV-1 protease is required for viral replication. The fully active viral protease targets specific proteolytic sites in $Pr160^{gag\text{-}pol}$ and $Pr55^{gag}$, proteolyses these precursor proteins, and finally produces RT, IN, MA, CA, and NC (Katz & Skalka, 1994, Luciw, 1996). Studies in the site-specific mutagenesis have demonstrated that noninfectious particles, containing uncleaved $Pr160^{gag\text{-}pol}$ and $Pr55^{gag}$ polyproteins, are produced if this enzyme is inactivated by mutation (Luciw, 1996).

Because HIV-1 protease is indispensable for viral replication, many efforts have been made to inhibit its enzymatic in the development of antiviral therapies (Deeks *et al.*, 1997). Currently, four protease inhibitors are used clinically either alone or in combination with RT inhibitors or other PR inhibitors, which are introduced at section 1.7 .

Reverse Transcriptase (RT)

Reverse transcriptase was first discovered in retroviruses. It is an RNA-dependent DNA polymerase, which allows HIV-1 itself to synthesize double-stranded DNA (cDNA) using viral RNA as the template. HIV-1 RT is a heterodimer composed of p51 and p66. It contains the activity of RNase H, which functions in reverse transcription by degrading the RNA moiety of RNA/DNA hybrids and thereby uncovering the template for viral DNA synthesis (reviewed in Luciw, 1996).

RT is processed in two steps from $Pr160^{gag\text{-}pol}$ precursor by viral encoded PR. Initially, protein p66, which consists of an N-terminus polymerase domain and a C-terminus RNase H domain, is cleaved from the central portion of $Pr160^{gag\text{-}pol}$ and forms a homodimer. Subsequently, a portion of the C-terminus is cleaved from one p66 subunit to produce a heterodimer of p66/p51. The N-termini of p51 and p66 are

identical, however, only subunit p66 retains the RNase H domain (reviewed in Luciw, 1996). The predicted structure of p66 has been compared to that of a clenched right hand, specific subdomains are designated as palm, thumb, and finger. A short connection sub-domain joins the p66 and p51. The viral RNA template and the tRNA primer are positioned within the palm position (reviewed in Coffin, 1996). Both domains of RT are indispensable for viral replication. Inhibitors of RT were the first prescription used in antiretroviral therapy. Although treatments with RT inhibitors are commonly associated with severe side effects and the emergence of resistant viral strains, RT inhibitors are still used for HIV-1 therapy because of their clinical value in combination with protease inhibitors.

Integrase (IN)

IN is about 32 kDa, and is proteolytically cleaved by PR from the C-terminus of $Pr160^{gag\text{-}pol}$ polyprotein. Genetic studies of viral mutants have shown that this viral enzyme is also essential for viral replication. The two principle functions of HIV-1 integrase are DNA cleavage and joining activities. After viral DNA has been generated by RT, IN assists in joining this double-stranded DNA covalently to the host DNA to form the provirus (reviewed in coffin, 1996).

1.2.3.3 Envelope Glycoproteins

HIV-1 envelope glycoproteins, including gp120 and gp41, are the products of highly variant *env* gene. They are expressed in the late stages of viral replication, and their expression is altered by the viral *rev* gene. The primary product of *env* gene is the envelope precursor gp160 glycoprotein that contains 850 amino acids. It is then cleaved by intracellular protease to yield an N-terminal gp120 subunit and a C-terminal gp41 subunit. Gp120 is designated the SU (surface glycoprotein) domain, which locates on the external surface of the virion envelope. Gp41 is designated the TM (transmembrane glycoprotein) domain, which traverses the virion envelope and severs as an anchor for gp120 (Veronese *et al.*, 1985). This envelope heterodimer is

shaped in a knob-and-socket-like formation (Figure 1. 4 & Figure 1. 5), and mainly functions for virus attachment and infection. These viral glycoproteins are responsible for the induction of syncytia in tissue culture cells. In addition, they serve as the major targets for the antiviral immune response of the infected host (Luciw, 1996; Levy, 1998).

Transmembrane glycoprotein: gp41

Gp41, the transmembrane (TM) subunit of envelope glycoprotein, is about 350 amino acids in length, contains 3 cysteine residues and four potential glycosylation sites, including fusion peptide, leucine zipper motif, transmembrane domain, and cytoplasmic peptide (Figure 1. 5). The first 20 amino acids at the N-terminus of gp41 are hydrophobic and define the fusion peptide. It is the intracellular portion of gp41, and required for the fusion of virion membrane with cell plasma membrane during the entry steps in viral replication (Luciw, 1996; Chang *et al.*, 1999). A second hydrophobic domain is the transmembrane domain of gp41, which spans the virion or cell membranes, serving as a bridge to link the cytoplasmic portion and intracellular portion of gp41. The region between fusion peptide and transmembrane domain is external to the membrane, and contains a highly conserved sequence predicated to be similar to the leucine zipper motif. Investigators have demonstrated that mutations in this region of gp41 block viral infectivity and cell fusion (Kliger *et al.*, 1997). Two α-helical regions, LLP-1 (for Arg826 to Leu854) and LLP-2 (for Tyr768 to Arg788) are predicted in the cytoplasmic domain of gp41. Synthetic peptides representing these two regions have shown the ability to induce the formation of pores in plasma membranes of tissue culture cells and cause cell lysis; therefore, these regions are designated as lentivirus lytic peptides (LLP) (Miller *et al.*, 1993; Luciw, 1996).

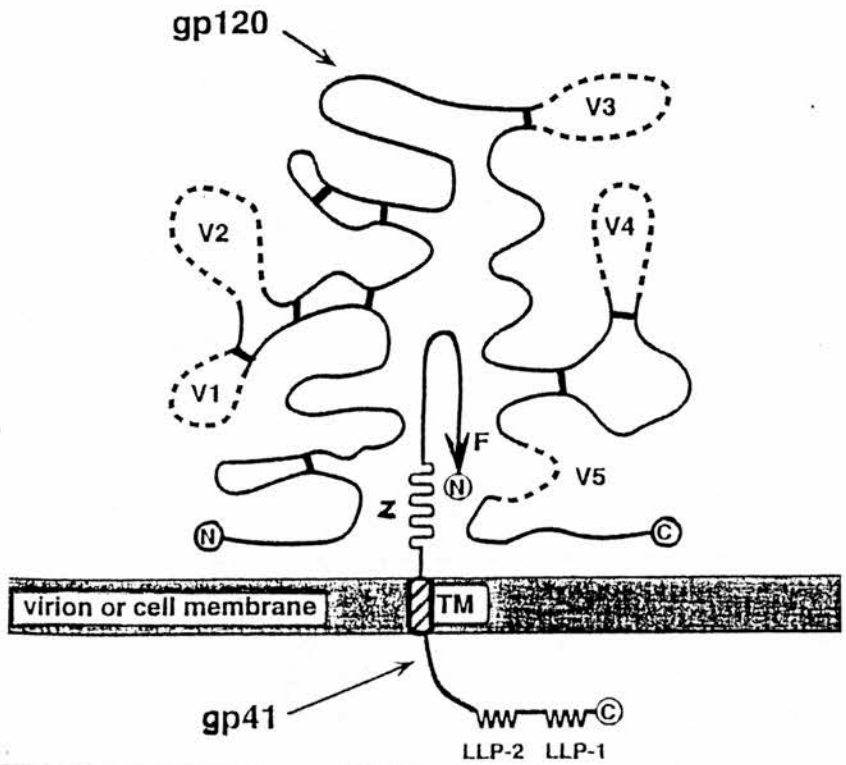


Figure 1. 5. Predicted folding pattern of HIV-1 envelople glycoprotein. Nine disulfide bonds have been identified within gp120 subunint, which are indicated by short connecting lines in the figure. Hypervariable domains are designated V1 through V5 and are drawn as broken lines. The predicted folding pattern and membrane association of the gp41 subunite are also shown. Regions labelled in the gp41 subunit are: **F**, fusion peptide (large arrow at N-terminus); **Z,** leucine zipperlike region (shown as a helix); **TM**, transmembrane domain (cross-hatched box); **LLP-1** and **LLP-2**, lentivirus lytic peptides 1 and 2 (shown as WW). Amino and carboxyl termini are labeled N and C, respectively, for both subunits. (Copied from *Fields Virology*, 1996)

Surface glycoprotein: gp120

Gp120 contains about 550 amino acids, involving 18 highly conserved cysteine residues. As an external surface subunit of HIV-1, the principal function of gp120 is interaction with cellular receptors, especially with CD4 receptor. This has been proved by previous studies, which employed antibodies to gp120 which successfully blocked CD4 binding (Moore & Ho, 1993; Levy, 1998).

Based on the biochemical analysis, a predicted model for the gp120 shows that about nine intra-chain disulfide bonds are formed between these cysteine residues (Luciw, 1996). The formation of these disulfide bonds delineates gp120 into five hypervariable regions (V1-V5) and five conserved regions (C1-C5) (Figure 1. 5). The actual function of each region is not yet entirely understood; however analyses of genetic mutations and molecular structure both provide evidence that each region of gp120 plays a role in the interaction with the cellular receptor (reviewed in Levy, 1998).

Investigations of the neutralizing antibody activity in HIV-1 infected hosts further confirmed that the majority of this neutralizing activity is specific for the V3 loop in gp120 (La-Rosa *et al.*, 1990; Robert-Guroff *et al.*, 1994; Levy, 1998). In *in vitro* studies, synthesized V3 peptides are capable of eliciting the virus neutralizing antibodies. Thus, the V3 region is designated as a principal neutralizing determinant (PND) of HIV-1 and believed to playing an important role in the early stage of infection (Luciw, 1996; Levy, 1998).

The V3 domain is the third hypervariable region of the gp120. It contains 35 amino acids arranged in a disulfide loop involving two cysteine residues, Cys301 and Cys336 (Figure 1. 6) (Milich *et al.*, 1993; Luciw, 1996). The nucleotide sequence of the V3 region shows great variability, however there are some relatively conserved sequences located either at the top of V3 loop (Gly317-Pro318-Gly319-Arg320-Ala312-Phe222) or near the Cys301 and Cys336 at the bottom of the loop (reviewed in Luciw, 1996).

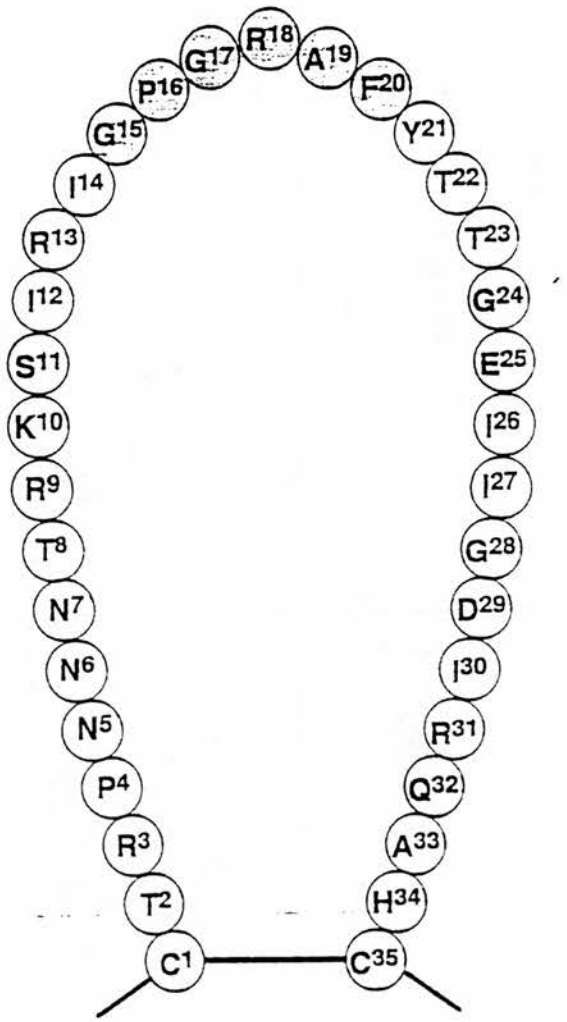


Figure 1. 6. V3 loop of HIV-1 envelope gp120. The one-letter amino acid code is used to identify residues in the consensus sequence for the V3 loop of HIV-1. The motif, G^{15}-P^{16}-G^{17}-R^{18}-A^{19}-F^{20} at the crown of the loop is relatively conserved between HIV-1 isolates. T-tropic SI viruses generally have a basic amino acid at least one of the positions at 11 and 25 and M-tropic NSI strains have an acidic amino acid or alanine at positions 11 or 25. (Modified from *Fields Virology*, 1996).

Mutagenetic studies demonstrated that point mutations within these conserved residues of the V3 loop might influence several properties of HIV-1. For example, some HIV-1 strains with mutations in Gly317-Pro318-Gly319 are not infectious and do not induce syncytium formation (de Jong *et al.*, 1992). Also, it has been found that mutations in the V3 sequence affect stabilisation of the association between gp120 and gp41 (Willey *et al.*, 1994; Luciw, 1996). Moreover, mutation of the conserved Cys336 at the base of the V3 loop precludes the cleavage of gp160 and binding to the CD4 receptor. Seemingly, this point mutation alters the overall tertiary loop structure of envelope glycoprotein and changes the conformation of the binding site for the CD4 receptor (Willey *et al.*, 1994). Apart from modulating affinity with the CD4 receptor, virus infectivity, and stability between gp120 and gp41, the V3 sequence has also been proved to hold the determinants for viral syncytium formation and cell tropism (Chesbro *et al.*, 1991; Milich *et al.*, 1993; Willey *et al.*, 1994; reviewed in Luciw, 1996). Single or multiple point mutations in amino acid Ser306, Ala321, Phe322, Gln328 of the V3 loop correlate with a slowly replicating non syncytium-inducing phenotype (NSI), whereas the original amino acids in these positions confer a rapidly replicating syncytium-inducing phenotype (SI) (Chesebro *et al.*, 1992; de Jong *et al.*, 1992; Fouchier *et al.*, 1992; Milich *et al.*, 1993). It is also documented that T-cell line-tropic viruses generally have a nonacidic amino acid at position Gln328, whereas macrophage-tropic viruses have an acidic amino acid at this position (de Jong *et al.*, 1992; Fouchier *et al.*, 1992; Milich *et al.*, 1993; Luciw, 1996).

1.2.3.4 Other Viral Genes and Their Products

As described previously, HIV genome contains six regulatory genes, including *tat*, *rev*, *vif*, *vpu*, *vpr* and *nef*, which encode Tat, Rev, Vif, Vpu Vpr and Nef viral proteins respectively. Tat and Rev are essentially regulatory proteins for RNA binding. Tat protein works for transcriptional initiation and elongation of viral RNA at the transcriptional level. Rev protein regulates splicing and transport of viral RNA from the nucleus to the cytoplasm at the post-transcriptional level (Cullen & Greene, 1990). Both Tat and Rev are very important for viral gene expression. They are

synthesised at both early and late stages of the life cycle of HIV-1, but are not packed into the virion (Cullen & Greene, 1990; Luciw, 1996). Vpu, Vif, Vpr and Nef are the accessory proteins of HIV-1. Only Vpr is found to be assembled into virions, others are not detected perhaps due to the small amounts in virus particles. Functions of these accessory proteins are not yet clear. Early studies of viral replication, based on tissue culture systems, showed that these gene products could be dispensed within certain HIV strains. Recently, more comprehensive studies performed *in vivo* suggested that Nef, Vif, Vpu and Vpr of HIV-1 play important roles in viral replication and HIV pathogenesis (reviewed in Miller & Sarver, 1997).

Transcription activator (Tat)

Tat is a product of the transcriptional transactivator (*tat*) gene, and about 14 kDa in molecular weight. It is encoded by two exons, the first coding exon is located in the central region of the viral genome between *vpr* and *env*; the second coding exon overlaps the translation frame for both *rev* and the gp41 domain in *env* (refer to Figure 1. 3). Tat is the major protein involved in up-regulating HIV transcription, and functions by interaction with a Tat-responsive element (TAR). TAR is an RNA loop structure formed in the 3' repeat R portion of the viral long terminal repeat (LTR). The interaction between Tat and nascent TAR RNA facilitates formation of an initiation complex for viral transcription and stabilization of the elongation complex. Genetic studies have demonstrated that when Tat is absent, initiation of viral transcription is low and transcriptional elongation is inefficient (reviewed in Luciw, 1996).

Recently, Tat has been found to be toxic to neurons and to induce cytokine production by glial cells *in vitro* (Jones *et al.*, 1998). They demonstrated that even a transient exposure of the brain tissue to Tat protein could result in profound and progressive neuropathological changes including an influx of inflammatory cells, gliosis, ventricular enlargement, and eventually lead to cellular loss and functional derangement of central nervous system (Jones *et al.*, 1998).

Regulator of Viral Expression (Rev)

Similar to the *tat* gene, the regulator of viral expression (*rev*) gene is also composed of two exons. The first coding exon is located in the central region of the viral genome between *vpr* and *env*; the second coding exon overlaps the translation frame for both *tat* and the gp41 domain in *env* regions (refer to Figure 1. 3). The *rev* gene encodes Rev protein, which has a molecular weight of 19 kDa and functions to regulate splicing and transport of viral RNA from the nucleus to the cytoplasm (Malim *et al.,* 1988; Luciw, 1996). For function, Rev requires a complex stem-looped mRNA, Rev-responsive element (RRE), which is located within the *env* gene immediately 3' of sequences encoding the junction of the gp120 and gp41 subunits (Malim *et al.,* 1992; reviewed in Luciw, 1996). The interaction between Rev and RRE functions to export unspliced or single spliced mRNA from nucleus to cytoplasm, which then act as templates for the translation of gag-pol or envelope precursor proteins. In the absence of Rev, no structural proteins can be made (Malim *et al.,* 1991; Luciw, 1996).

Viral Infectivity Factor (Vif)

The vif protein of HIV-1 is encoded by *vif* gene, which is located immediately downstream of the *pol* gene (refer to Figure 1. 3). Vif contains 193 amino acids, has a molecular weight of 23 to 27 kDa, and presumably influences the infectivity of cell-free virions. It is produced in the late stage of viral replication cycle, and is observed to accumulate in the cytosol and cytoplasmic membrane fractions of infected cells (reviewed in Luciw, 1996).

The HIV-1 Vif protein is hypothesised to play a role in the early events in viral replication. Liu *et al.* demonstrated that abundant Vif molecules were present in intact virus particles (Liu *et al.,* 1995). It was observed that these Vif proteins were associated with intermediate filaments in the cellular cytoskeleton which directly connect the plasma membrane with the nuclear membrane. The association and interaction between Vif proteins and intermediate filaments are thought to provide an additional mechanism for transporting incoming virions to the nucleus (Karczewski

and Strebel, 1996). Also, Vif was observed to stabilise newly synthesised virion DNA intermediates during virion formation in the virus-producing cells (Fouchier *et al.,* 1996; Simon and Malim, 1996). However, all these observation need further investigations.

Viral Protein R (Vpr)

Vpr is a 14kDa virion-associated protein and so far, it is the only accessory viral protein found to assemble into HIV-1 virions. It is encoded by *vpr* gene, which locates between the end of *vif* gene and the beginning of the first exon of *tat* gene (refer to Figure 1. 3).

Two principle functions of Vif have been identified recently, both related to the presence of Vpr in the intact virion. The first, Vpr has been observed to assist in targeting of the viral preintegration complex (PIC) to the nucleus (Heinzinger *et al.,* 1994; Yao *et al.,* 1995). Entry of the PIC into the nucleus is an essential step in retrovirus replication and is required for subsequent integration of the proviral genome into host DNA. Unlike other retroviruses, HIV has developed specific mechanisms for the PIC nuclear import, which are mediated by Vpr and MA independently via distinct nuclear localisation signal sequences (NLS) (Heinzinger *et al.,* 1994; Yao *et al.,* 1995). Cultured cells infected with viral particles with double mutations in the NLS of both MA and Vpr showed in a reduction of viral replication (Miller and Sarver, 1997).

The second function of Vpr is the arrest of dividing cells in G2 phase of the cell cycle. Generally, the cell cycle involves four major phases, G1 (first gap), S (DNA synthesis), G2 (second gap) and M (mitosis) sequentially. In *in vitro* culture system, it was found that the cytostatic function of Vpr caused cells to stay in the G2 phase (DiMarzio *et al.,* 1995; Jowett *et al.,* 1995; Bartz *et al.,* 1996). Experiments estimated that HIV-1 infected cells arrested in G2 phase spend an average of 20 hours producing virus whereas cells progressing normally through the cell cycle spend only three hours in optimal virus production, thus arrested cells displayed an

increase of virion production (Miller and Sarver, 1997). Moreover, with the presence of Vpr, some cells that arrest in G2 phase returned to S phase instead of progressing to M phase, and thus the virus production is further enhanced (Bartz *et al.*, 1996).

Viral Protein U (Vpu)

The Vpu protein is encoded by *vpu* gene. This reading frame is unique to HIV-1, which is located from the 3' end of first *tat* and *rev* exon to the 5' end of *env* gene (refer to Figure 1. 3). HIV-1 Vpu protein is about 9.2 kDa in molecular weight, and is a multifunctional phosphoprotein composed of two distinct protein domains, which are the N-terminal hydrophobic domain and the C-terminal hydrophilic cytoplasmic domain. Immunolocalisation studies revealed that Vpu is usually present in the perinuclear membranes of HIV-infected cells, including endoplasmic reticulum (ER) and Golgi-bodies (Strebel *et al.*, 1989; reviewed in Miller and Sarver, 1997).

Two biological functions of Vpu have been identified recently. The first is the degradation of the CD4/gp160 complexes. This CD4/gp160 complex is formed in the ER system during the course of HIV infection. Vpu appears to recognise specific sequences in the cytoplasmic domain of CD4 and bind to the CD4/gp160 complex. The interaction between Vpu and CD4 then triggers the degradation of CD4 and releases gp160. Once released from the complex, gp160 is processed to gp41 and gp120, which construct the virion envelope. It has been found that the phosphorylation of the cytoplasmic domain of Vpu is absolutely essential for this degrading pathway (Paul *et al.*, 1998). The other function of Vpu is to enhance the release of HIV-1 particles from the surface of infected cells (Paul *et al.*, 1998). It has been observed that *vpu*-deficient virions are released poorly from infected cells *in vitro*. Although the actual molecular mechanism is not conclusive yet, it is believed that Vpu is critical in virus release (Paul *et al.*, 1998).

Negative Factor (Nef)

HIV-1 Nef protein is encoded by *nef* gene (refer to Figure 1. 3), and its molecular weight is about 27 kDa. During the early stage of virus replication, Nef is abundantly synthesised in the cytoplasm, becomes myristoylated and then associated with the plasma membrane of infected cells. The name, negative factor, was given when Nef was thought to be a down-regulator of viral gene expression. Subsequently, according to the discoveries that infection with nef-defective virus is usually associated with low levels of viral replication and absence of clinical disease in animal models (Kestler *et al.,* 1991), and the Nef-defective HIV-1 strain was identified in a naturally occurring human cohort of long-term non-progressors (Deacon *et al.,* 1995), Nef protein is now known to play a prominent role in HIV replication (Vicenzi *et al.,* 1997), and pathogenesis (Mangasarian & Trono, 1997; reviewed in Miller and Sarver, 1997).

Recently, Nef has been observed to downregulate the expression of CD4 from the surface of infected cells in cell culture systems. Also, it has been found that Nef protein might enhance the infectivity of cell-free virus (Guatelli, 1997). However the relevance of these findings to HIV pathogenesis *in vivo* requires further investigation.

1.3 Life Cycle of HIV-1 Replication

The replication cycle of HIV-1 can be simply divided into an early and a late phase. The early phase begins with attachment of a virion to a cell surface receptor and continues to the formation of a provirus integrated into the host cell genome. The late phase begins with transcription and processing of viral RNA from the integrated proviral template and ends with release of progeny virions from the infected cell.

1.3.1 Virion Attachment and Entry

At least two cell surface receptors are now known to be involved in HIV-1 entry to cells. They are the CD4 and chemokine receptors.

1.3.1.1 Interaction with the CD4 Receptor

The discovery of the CD4 molecule as the major cellular receptor of HIV-1 was an early and important step in the understanding of HIV-1 infection. CD4 is a member of the immunoglobulin superfamily and functions primarily as a coreceptor in antigen-MHC II dependent interactions. The structure of the N-terminal extracellular portion of CD4 appears to be a rod-like monomer consisting of four immunoglobulin-like domains (D1 to D4). This extracellular portion is anchored to the cell surface by a transmembrane region followed by a short cytoplasmic tail at the C-terminus (Janeway & Travers, 1996). Studies in electron microscopy and X-ray crystallography reveal that direct fusion of the viral and cell plasma membranes should be the principle pathway for virion entry, which is initiated by virion attachment (Wang *et al.*, 1990; Ryu *et al.*, 1990; Luciw, 1996).

The CD4 molecule is identified as the major receptor responsible for HIV-1 attachment through an interaction with gp120. By means of biochemical and genetic analyses, the first immunoglobulin-like domain (D1) of the CD4 molecule has been recognized as the major binding site for the viral surface envelope protein gp120 (Broder *et al.*, 1993; Brand *et al.*, 1995; Sullivan *et al.*, 1996). The CD4 binding region on gp120 appears to be a complex folded structure with discontinuous sites

from several regions coming in contact with CD4 molecule (Moore & Ho, 1993). This interaction is observed to involve primarily two hydrophobic domains in the conserved C2 and C4 regions of gp120, and probably other unspecified regions. Both conformations of CD4 and gp120 are very important for virus binding, and will affect virus entry (Moore & Ho, 1993).

The high affinity binding of the HIV-1 Env proteins to the CD4 molecule appears to induce conformational changes in CD4-gp120-gp41 complex (Figure 1. 7). The actual mechanism is not yet clear. In a prevalent model, it is believed that interaction of CD4 and other surface receptors, such as CCR5 or CXCR4 with HIV-1 gp120 promotes dissociation of the gp120 subunit from the gp41 subunit in virion membranes (Moore & Ho, 1993; Luciw, 1996; Levy, 1998; Doranz *et al.*, 1999). This dissociation results in exposure of the hydrophobic fusion peptide at the N-terminal of gp41 (Figure 1. 7). After exposure, this fusion peptide integrates into the lipid bilayer of the cell plasma membranes. Subsequently, the viral and cell membranes fusion are fused. The entry process then continues with formation of the fusion pore and transfer of the viral nucleocapsid complex into the cell interior thus initiating the infection cycle (Luciw, 1996; Kliger *et al.*, 1997; Levy, 1998; Chang *et al.*, 1999; Doranz *et al.*, 1999).

Though CD4 was found to be the major receptor of HIV-1, further studies have shown that the CD4 receptor is not sufficient on its own for virus entry. There is evidence from the studies of Kikukawa *et al.* that several human cells expressing high levels of CD4 molecule cannot always be infected by HIV-1 (Kikukawa *et al.*, 1986). Also, Tersmetta *et al.* demonstrated that in some animal models, although human CD4 molecules were successfully expressed on the cell surface, these cells remained resistant to HIV-1 infection (Tersmette *et al.*, 1989a). Moreover, Rey *et al.* found that productive infection of CD4+ cells by certain HIV strains was not blocked by anti-CD4 antibodies (Rey *et al.*, 1991). Taken together these findings suggest that other cell surface factors are involved in HIV-1 entry.

1.3.1.2 Alternative Factors

The discovery of chemokine receptors was another important step in understanding the pathogenesis of HIV-1 infection. In searching for accessory receptors for HIV infection, a human cDNA clone that expressed a protein, fusin, was found capable mediating fusion in the mouse-human CD4+ cell model. When introduced into HIV-resistant mouse cells expressing human CD4 molecules, this fusion-inducing molecule, fusin, rendered those HIV-resistant cells susceptible to HIV infection (Feng *et al.,* 1996). This molecule, now termed CXCR-4, has been proved to act as a coreceptor for T-cell-line tropic HIV-1 strains, which plays a critical role in T-tropic virus fusion and entry into permissive cells (Feng *et al.,* 1996; Berger, 1997; Berger *et al.,* 1999).

CXCR4 is a 46 kDa integral membrane glycoprotein and a member of the α-chemokine receptor family within the superfamily of G-protein-coupled seven-transmembrane-domain receptors (Feng *et al.,* 1996). The natural ligand for CXCR4 is stromal cell-derived factor 1 (SDF-1). SDF-1 is a member of a family of low-molecular-weight cytokines called CXC-chemokines that mediate inflammation by recruiting immune cells to the site of injury (Bleul *et al.,* 1996; Feng *et al.,* 1996; Oberlin *et al.,* 1996). CXCR4 has been shown to act as a coreceptor for T-tropic, but not for macrophage-tropic HIV-1 strains.

Some investigators also found that the presence of certain β-chemokines including RANTES (regulated-upon-activation normal T expressed) and macrophage inflammatory protein-1α (MIP-1α), and MIP-1β in the cell culture supernatant inhibit cell:virus membrane fusion and the entry of macrophage-tropic or NSI HIV-1 strains, however these β-chemokines are insensitive to T-cell-line-tropic or SI strains (Deng *et al.,* 1996; Dragic *et al.,* 1996). More chemokine receptors, mainly CCR3 and CCR5, were subsequently identified as the coreceptors responsible for the entry of macrophage-tropic, NSI HIV-1 strains (Alkhatib *et al.,* 1996; Choe *et al.,* 1996; Berger, 1997).

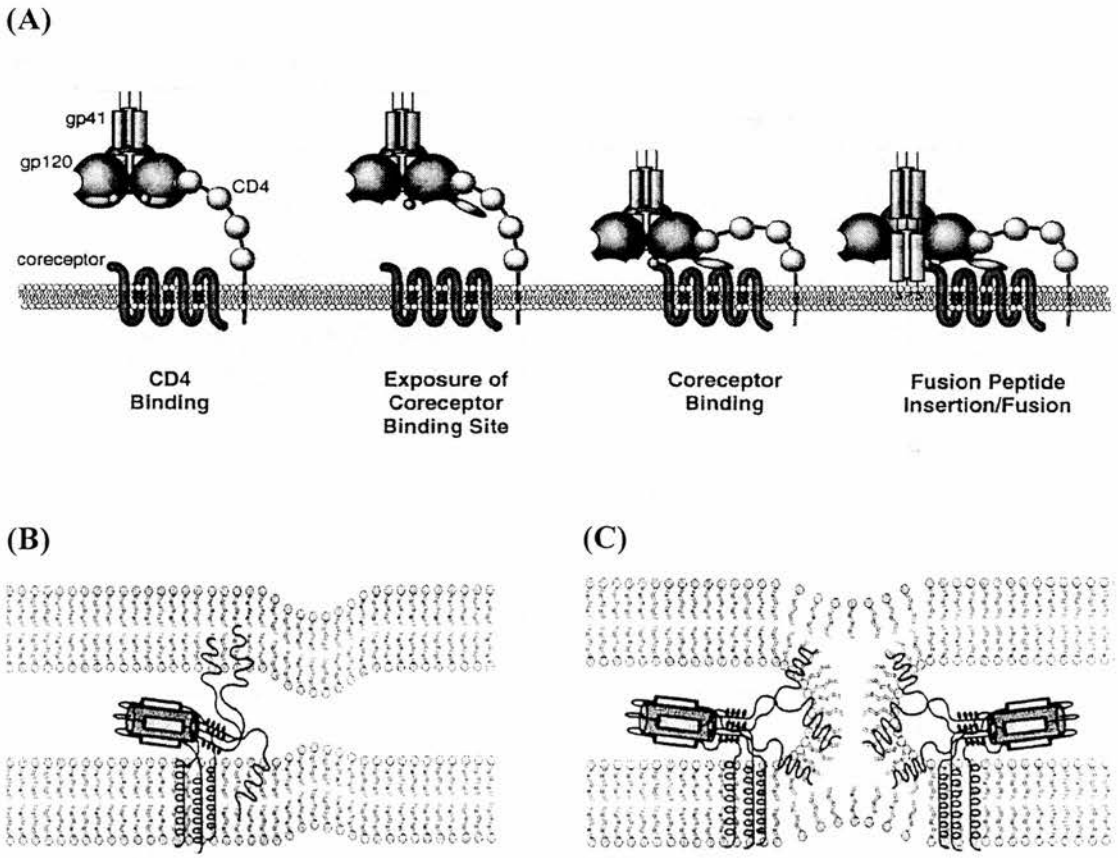


Figure 1. 7. Model for virion attachment and entry.

(A) HIV entry begins when the gp120 subunit of Env contacts CD4 on the surface of a cell (far left). CD4 binding induces a rapid and reversible conformational change in gp120 that exposes a binding site for coreceptor. Binding to a coreceptor brings the virus in close proximity to the cellular membrane and induces a conformational change in the gp41 subunit of Env. The change in gp41 exposes the fusion peptide of Env gp41 (far right). (Modiefied from Doranz *et al.*, 1999).

(B) Subsequently, the exposed fusion peptide inserts into, and destabilizes, both the target and viral membranes primarily as a helix. Insertion of the fusion peptide facilitates mergence of these two membranes. (Modified from Chang *et al.*, 1999).

(C) After target and viral membranes merge, several units of gp41 fusion peptide assemble to form the fusion pore to allow the flow of nucleocapsid of the virus. (Modified from Chang *et al.*, 1999).

Further evidence that CCR5 could be an essential co-receptor for HIV-1 infection was shown in studies of cells with defective CCR5 receptors. Several individuals who remained free from infection with HIV despite repeated exposure through sexual intercourse with HIV-positive partners were reported by Paxton *et al.* (Paxton *et al.,* 1996). Subsequently, a mutant allele of *CCR5* gene that contains a homozygous 32-base-pair deletion was identified from the exposed but uninfected individuals. This deletion inhibits the expression of CCR5 receptor on the cell surface, and presumably results in resistance to HIV-1 infection (Liu *et al.,* 1996; Samson *et al.,* 1996; Quillent *et al.,* 1998). Similar to α-chemokine receptors, the β-chemokine receptor also belongs to the G-protein-coupled seven-transmembrane-domain family. Without these receptors, the *env*-mediated membrane fusion at the early binding steps might be inhibited and result in blocking HIV-1 infection, however this do not appear to inhibit virus production in chronically infected cells (Oravecz *et al.,* 1996; Levy, 1998). Although the details remain to be resolved, the sequence of the V3 loop is an important determinant of which receptors are used for viral entry (Cocchi *et al.,* 1996; Wu *et al.,* 1996; Moore, 1997; Farzan *et al.,* 1998).

Other co-receptors have also been discovered, including CCR-2b, Bonzo/STRL33, BOB/GPR15, but how they interact with virus infection requires further investigation (Deng *et al.,* 1997; Frade *et al.,* 1997; Liao *et al.,* 1997).

1.3.2 Integration of Viral DNA

A pre-integration complex is produced due to virion capsid rearrangement soon after the HIV-1 nucleocapsid gains entry into the cytoplasm of the host cells (Farnet & Haseltine, 1991). Subsequently, the synthesis of proviral DNA is activated by reverse transcriptase (RT). The newly synthesized complementary viral DNA is then incorporated into the host genome with the help of viral integrase (IN), and the HIV-1 life cycle goes into the provirus stage.

1.3.2.1 Mechanism of Reverse Transcriptase

For the purpose of incorporation of the viral genome into the host cell genome, single-strand viral RNA requires first to be transcribed into complementary double-strand DNA. This transcription is generated by the virally encoded RT enzyme.

At both the 3' and 5' ends of HIV-1 RNA genome, there exist a short repeated RNA sequences (R), which belongs to LTR region. Initially, RT activates the synthesis of the first DNA strand (minus strand). The procedure starts from a $tRNA^{lys}$ primer hydrogen-bonded to the primer-binding site (PBS), which located near the 5' end of the viral RNA genomic template. A short DNA copy is first made consisting of R and U5 regions lying between PBS and the 5' end of the viral genome. This fragment is designated as the strong-stop DNA of the minus strand (Figure 1. 8). Once the strong-stop DNA is produced, the RNase H of p51/p66 RT heterodimer is activated and degrades RNA in the DNA/RNA hybrid. The degradation results in exposing the newly synthesized R' region of the strong-stop DNA which is complementary to the short repeated R region at the 5' end of the viral RNA genome. This strong-stop DNA then jumps to the 3' end of viral genome, and the exposed R' region hybridizes to complementary sequences in the R region at the 3' end of the viral RNA genome. After the strong-stop DNA primes, the minus strand DNA is extended completely by RT to the 5' end of PBS, since R and U5 have been removed at the beginning. As the minus strand DNA elongates, the RNA template is gradually degraded by the RNase H of RT. Therefore, the synthesized minus strand DNA can serve as the template for synthesis of plus strand DNA (reviewed in Coffin, 1996, and Luciew, 1996).

The synthesis of plus strand DNA utilises two primers, poly-purine track (PPT) that borders the U3 region and central PPT (cPPT) that nears the *pol* gene of the minus strand DNA template. The elongation of plus strand DNA is started in parallel from PPT and cPPT primers. One elongation generated by the PPT primer is extended toward the end of the minus strand DNA template to produce strong-stop DNA of the plus strand DNA, which functions the same as minus strand strong-stop DNA. Once produced, this strong-stop DNA will then jump and hybridize to

complementary sequences in the PBS region at 3' end of the minus strand DNA template, and continues to elongate the plus strand DNA to the central termination signal (CTS) that nears cPPT site (Wainberg & Gu, 1995; Luciw, 1996). At the same time, another DNA elongation initiating at the cPPT site is completed by RT to the end of the minus strand DNA template. These synthesized fragments are then linked together to yield the completed double-stranded linear DNA which contains the LTR (U3-R-U5) at each end.

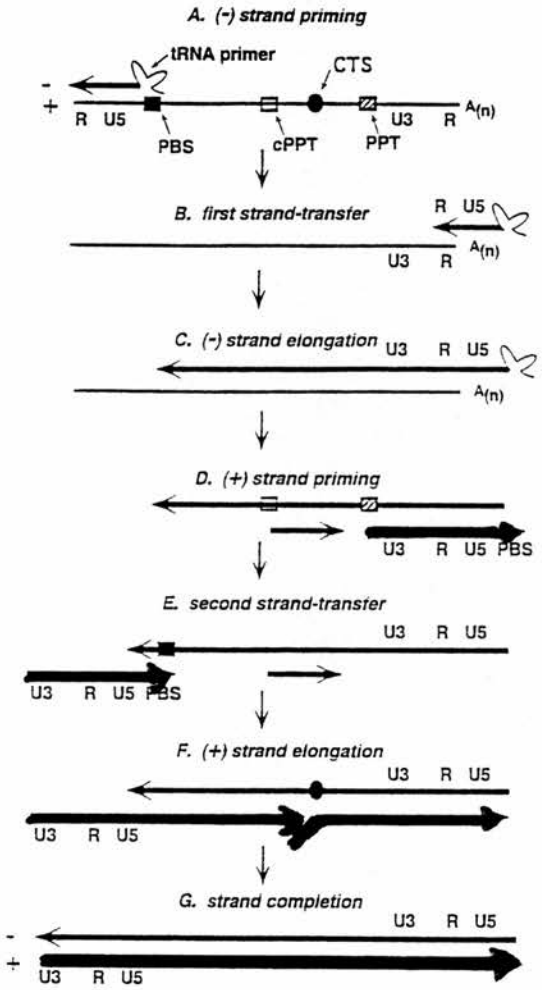


Figure 1. 8. Mechanism of reverse transcriptase. The viral RNA genome is shown as a thin solid line, designated (+). The poly-A tail on viral RNA is labelled A(n). The first strand of DNA is shown as a heavy solid line, designated (-), which is primed by a $tRNA^{lys}$. Synthesis of the second DNA is primed by a short viral RNA fragment generated by RNase H activity at the PPT that borders U3 and cPPT. The second DNA strand is represented as thick solid line. Details were described in text. (Copied from *Fields Virology*, 1996).

1.3.2.2 Integration of Proviral DNA

Following viral DNA synthesis, the pre-integration complexes containing linear synthesised DNA, IN and other viral proteins are formed and translocated into the nucleus of the host cell. The entry of this pro-integration complex initiates viral integration. During the process of translocation, two bases at the either 3'end of viral DNA are cleaved by IN, leaving a 3'OH end. The ends thus have the sequence

$^{5'}$ **AATG.....CA-OH** $^{3'}$
$^{3'}$ **HO-AC.....GTAA**$^{5'}$

This modified viral DNA joins into the host DNA by a strand transfer reaction. The strand transfer reaction simultaneously joins the 3'ends of viral DNA to host genomic DNA through a direct attack on a phosphate group by hydroxyl group of the 3' end of viral DNA; however the unaltered 5'ends of the viral DNA remains unjoined. A cellular DNA repair system fills in the resulting gap in the molecule, displacing two mismatched bases at the 5'end of the viral DNA and ligating the remaining ends. Thus, viral DNA successfully integrates into the host genome and initiates the provirus stage. Subsequently, proviral DNA may replicate and survive as long as the host cell is alive. At this stage, the viruses stay latent or co-exist with the host genome in a non-productive stage until unknown factors trigger off a productive stage. When a provirus goes into a productive stage, proviral DNA starts to express and produce the viral components. After viral assembly is completed, the host cell will be destroyed and progeny viruses will be released (reviewed in Coffin, 1996, and Luciw, 1996).

1.3.3 Expression of Viral Products

Viral RNA synthesis occurs in the cell nucleus and uses the integrated provirus DNA as the template. This process is complicated, and involves several cellular proteins and viral regulators. HIV-1 proviral DNA contains two identical LTRs at 3' and 5' end, but they function differently. 5' LTR works for the initiation of viral RNA synthesis, whereas 3' LTR works for ending transcription (Luciw, 1996). The U3 domain of HIV-1 5' LTR contains basal promoter elements, including a TATAA

box for initiation by host cell RNA polymerase II, and sites for binding the cellular transcription factor SP1. Initiation of viral RNA synthesis occurs at the U3/R border of the 5' LTR. First of all, an initiation complex for full-length viral RNA transcription is formed through interaction between HIV-1 Tat and nascent TAR RNA. Subsequently, an R-U5 segment is transcripted and serves as the leader sequence for RNA elongation. The 5'ends of the transcripts are post-transcriptionally capped with 7-methylguanosine by cellular enzymes soon after elongation initiates. HIV-1 RNA is synthesised by cellular RNA polymerase II and the 3' end of the viral transcript is terminated at the border of R/U5 in the 3' LTR. Signals in U3 region are recognised by cellular enzymes that add poly-A tails at the 3' ends of viral transcript (Barré-Sinoussi, 1996; Luciw, 1996). After transport to the cytoplasm, a fraction of these full-length RNA copies is reserved as the genomic RNA that is eventually assembled into progeny virions. The others serve as precursors for alternatively spliced mRNAs that are translated in the cytoplasm to produce viral proteins (Barré-Sinoussi, 1996). In general, this gene expression is regulated by *tat* and *rev* genes (refer to section 1.2.3.4).

1.3.4 Virion Assembly

The first event in virion assembly is presumed to be the production of an assembly of intermediate nucleoprotein complexes, which are composed of gag-pol precursor polyprotein ($Pr160^{gag\text{-}pol}$), $Pr55^{gag}$ and genomic viral RNA. Subsequently, as described previously, viral protease is activated to generate the proteolytic processing of Gag, Gag-Pol and Env precursor polyproteins. Cleavage of these polyproteins produces a mature nucleocapsid composed of fully processed *gag* (MA, CA, NC, p6, p1 and p2) and *pol* gene products (PR, IN, RT) as well as two molecules of the viral single-stranded RNA genome. This mature nucleocapsid then migrates to a budding site in the plasma membrane. Finally, oligomers of Env glycoproteins are also inserted into the budding site in the plasma membrane. Under the interaction between the cytoplasmic tail of the gp41 subunit in these oligomers and the MA domain of $Pr55^{gag}$ in the newly formed viral nucleocapsid, viral particles are extruded, or budded through the plasma membrane. The life of the host cell ends with release of new virions (reviewed in Barré-Sinoussi, 1996).

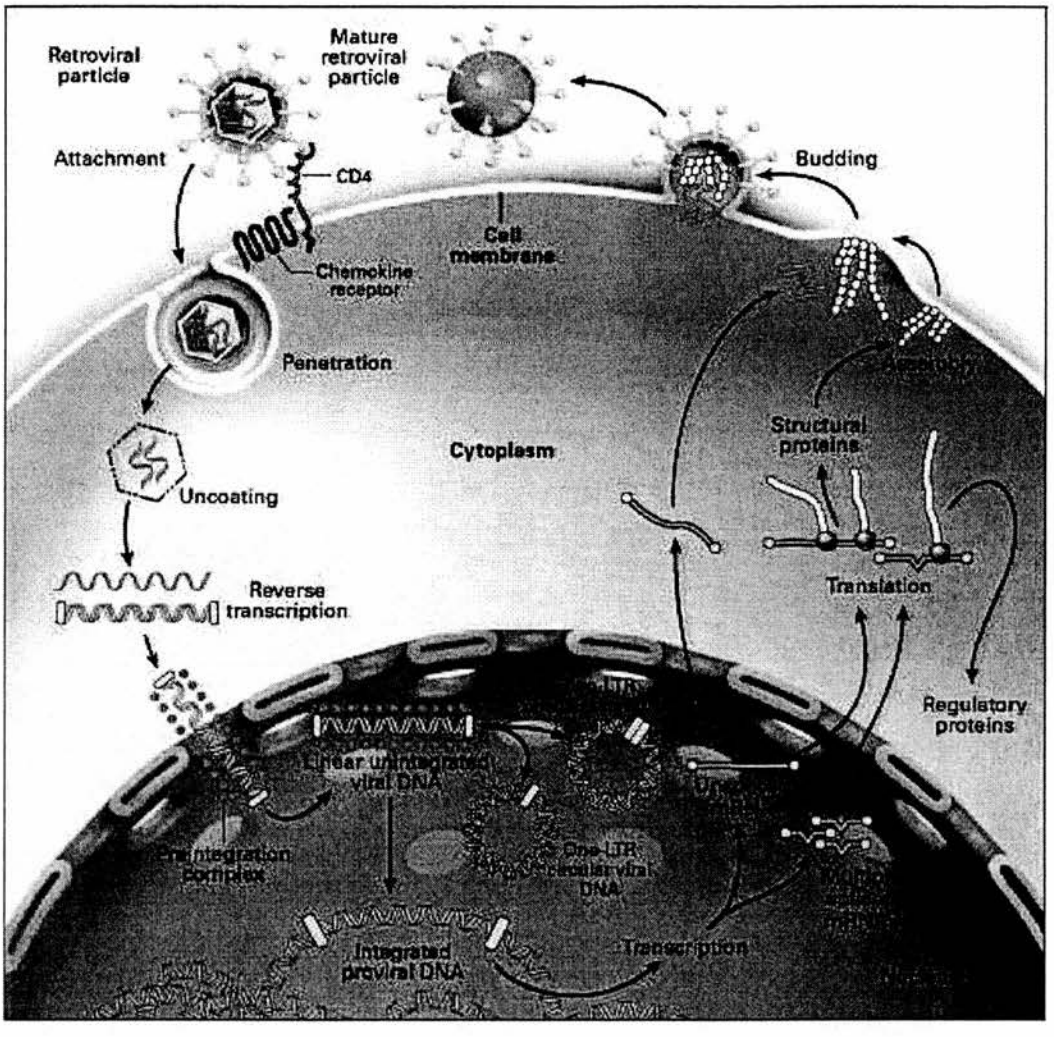


Figure 1. 9. Overall life cycle of HIV-1. (From Furtado *et al.*, 1999).

Early stage: HIV virions enter host cells after binding to the cellular receptors (refer to Figure 1. 7). The HIV-1 RNA genome then enters the cytoplasm as part of a nucleoprotein complex. The viral RNA genome is reverse-transcribed into a collinear DNA duplex, which has terminal duplications (LTRs). Soon as the viral DNA synthesised, the linear viral DNA molecule is incorporated into a preintegration complex that targets to the nucleus. In the nucleus, most of the viral DNAs are integrated into the host genome, although some remain unintegrated.

Late stage: Production of new virus particles is initiated by the transcription of new viral RNAs. Both full-length unspliced viral mRNA and spliced viral mRNA are synthesised. A fraction of the unspliced viral RNAs are translated into the viral structural precursor proteins, and others are reserved as the viral genomic RNA. The spliced mRNAs are translated into the viral accessory/regulatory proteins. All the viral transcripts are exported into the cytoplasm, where translation and assembly and processing of the retroviral particle take place.

This cycle is completed by the release of infectious retroviral particles from the host cell.

1.4 Genetic Diversity of HIV-1

A major problem for HIV-1 researchers has been the extraordinary ability of HIV-1 to undergo genetic variation. HIV-1 has been cloned and sequenced by a number of independent groups and a large number of nucleotide differences were detected. These studies have reported that a high degree of genomic heterogeneity in HIV-1 was present between and even within patients (reviewed in Barré-Sinoussi, 1996). This observation led to the term "quasispecies" to describe a group of closely related but genetically distinct viral variants (Goodenow *et al.*, 1989). It has been postulated that mutation and perhaps genomic recombination are the major factors responsible for this viral variation (Luciw, 1996).

Genetic variability is determined by the mutation rate per replication cycle, the number of replication cycles within a fixed time, and the selective advantage or disadvantage of a particular variant. The life cycle of HIV-1 involves several enzyme systems during replication, which is complex and thus provides many opportunities for mutation. Among these enzyme systems, reverse transcriptase that facilitates proviral DNA synthesis involving two strand-transfer or strand-jump steps is believed to play an important role in genomic mutation (Coffin, 1996; Luciw, 1996). The fidelity of HIV-1 RT is low. It has been reported that the error rate of HIV RT was 1/1700 (Roberts *et al.*, 1988) to 1/4000 (Preston *et al.*, 1988), equal to 5 to 10 errors per genome per round of replication. In addition, frame shifts, deletions, insertions and recombination events might also increase the infidelity of RT (Katz & Skalka *et al.*, 1990; Coffin, 1995; Temin, 1993). Also, some investigations demonstrated that viral RNA polymerase II might cause errors to be incorporated in genomic RNA during synthesis. Mansky *et al.* estimated recently that the mutation rate of HIV-1 was 3.4×10^{-5} mutations per base pair per replication cycle (Mansky & Temin, 1995). Thus, a high mutation rate combined with the extremely high turnover rate of HIV-1, which is estimated in the order of 10^9 virions produced per day, provides the basis for the continual emergence of new virus variants (Barré-Sinoussi, 1996).

Further evidence suggests that the high sequence variability of HIV-1 may be also due to selection by the host immune system. It has been known for some time that individual HIV-1 genes differ in their variability. Generally the *env* gene is much more diverse than *gag* or *pol* genes. Within the *env* gene, V1/V2 and V3 domains show much higher variability than other regions. These super-variable domains function as the targets for neutralizing antibodies. In order to escape from antibody neutralization or cytotoxic T cell elimination, the outstanding diversity of these surface domains is very advantageous (Barré-Sinoussi, 1996).

At a functional level, these various selective forces imposed by the host immune response may generate HIV-1 variants, which differ in their tropism for cells of different lineage. While the CD4 molecule is ordinarily required for efficient entry of most HIV-1 variants, some strains are able to infect macrophages and others show a preferential tropism for T cell lines. Macrophage-tropic viruses are more effective at invasion across a mucosal barrier, however subsequent *in vivo* evolution may lead to the emergence of more T-cell tropic variants. This suggests that diversity of HIV-1 may facilitate infection of different cell types (reviewed in Barré-Sinoussi, 1996).

Recombination between HIV-1 genomes has been reported, and this may also attribute to genomic diversity (Robertson *et al.,* 1995). The process of recombination could lead to new viruses being produced with different biologic and pathogenic properties. Moreover, it could introduce new strains into the population thereby challenging vaccine approaches. Between HIV-1 strains, recombination appears to occur most often within the *gag* and *env* regions (Cornelissen *et al.,* 1996; Kampinga *et al.,* 1997). The extent of recombination between strains is not yet clear, but it is believed that the natural frequency may be very high (Robertson *et al.,* 1995).

1.5 Clinical Aspects of HIV-1 Infection

1.5.1 Natural History of HIV-1 Infection

Generally, infection with HIV-1 will initiate an acute phase characterised by a transiently high viral load. The symptoms in the acute phase are minor, and many infected individuals do not even know that they have been infected. In some cases, a flu-like seroconversion illness might also occur within 1 to 4 weeks. The symptoms of primary infection, including headache, retro-orbital pain, muscle aches, sore throat, low-grade or high-grade fever and swollen lymph nodes normally last for 1 to 3 weeks, though lymphadenopathy, lethargy and malaise can persist for many months. Antibodies against the core or envelope viral proteins can be detected, usually six to eight weeks after initial infection (reviewed in Feinberg, 1996; Levy, 1998).

Following acute phase, a sharp reduction of free virus in the blood will then ensue, and HIV-1 infections go into an "asymptomatic" period. This asymptomatic phase can last from several months to more than 10 years. During this phase, physical examination might show no obvious abnormality, but about one-third of patients have persistent generalised lymphadenopathy (PGL) (reviewed in Feinbery, 1996; Levy, 1998).

As the disease progresses, the amount of infectious virus and the number of infected cells gradually increase, but the number of CD4+ lymphocytes declines. Later in infection, patients develop symptoms and abnormalities consequent on the low level of CD4+ T cells. This is known as the symptomatic phase or acquired immune deficiency syndrome (AIDS) commonly associated with immune abnormalities, opportunistic infections, neurological disorders, and unusual forms of cancer (Schulz *et al.*, 1996).

1.5.2 Diagnostic Techniques

HIV infection is commonly diagnosed by detecting antibodies specific to the virus or by detecting the virus itself (Table 1. 2).

Table 1. 2. Criteria for HIV infection for persons aged more than 13 years

(From *MMWR*, CDC, 1992)

- Repeatedly reactive screening tests for HIV antibody (e.g., enzyme immunoassay) with specific antibody identified by the use of supplemental tests (e.g., Western blot, and immunofluorescence assay).
- Direct identification of virus in host tissues by virus isolation
- HIV antigen detection
- A positive result on any other highly specific licensed test for HIV

1.5.2.1 Immunoassays

Antibodies to HIV are undetectable in the first six to eight weeks after infection. This period is known as the diagnostic window or serologic latency. Subsequent to serologic latency, antibodies to HIV-1 viral envelope glycoproteins, IN, RT, and *gag* gene products are produced (Gürtler, 1996).

To detect viral antibodies, enzyme-linked immunosorbent assay (ELISA) and agglutination assays are usually used. Routinely, ELISA assays are employed for screening, follow by a confirmatory assay. Usually, the immunoblot assay (Western Blot) is employed for confirmation, and has become the gold standard for investigating reactive results of ELISA screening (Gürtler, 1996). A double-antigen ELISA assay is frequently used in the early phase of seroconversion, because it is more sensitive and more specific than any other assay (Constantine *et al.,* 1993). However, the higher specificity of this assay may cause a poor ability to capture various antibodies against variant HIV strains. The possibilities of false-negative results need to be taken into consideration (Schable *et al.,* 1994).

1.5.2.2 Nucleic Acid Based Assays

Nucleic-acid-based assays, such as the Polymerase Chain Reaction (PCR) are employed to achieve genomic level testing for HIV. These assays are extremely sensitive and specific; moreover, they may detect free RNA virus particles by reverse- transcript PCR (RT-PCR) method. These tests are likely to be particularly useful in the serological diagnostic window period (Gürtler, 1996).

Usually, nucleic-acid-based assays are more expensive and not as convenient as the methods described above. They are not always necessary in the primary diagnostic procedure. Nevertheless they are of considerable use in more detailed investigations, for example, to determine the infection status of newborn babies of infected mothers, to subtype HIV variants and to identify various HIV strains for forensic reasons. They are also used to sequence viral enzymes, such as reverse transcriptase or protease genome fragments, for monitoring drug resistance and therapeutic efficacy (Gürtler, 1996).

1.5.3 Classification System for HIV Infection

After the discovery of the infectious agent of AIDS, numerous terms were used to describe the symptoms, signs, and laboratory findings of HIV infection. It is important to have a universal classification system for HIV infection and AIDS. Categorising the clinical conditions and stages of HIV infection makes it easier to compare directly reports from different centres and countries on clinical trials and other research projects. Therefore, in 1986, the Centers of Disease Control in the USA proposed a classification system on the basis of clinical disease criteria (CDC, 1986). Aetiological studies later confirmed that the primary target of HIV was CD4+ T-lymphocytes. Also, studies in the natural history of HIV infection discovered that the loss of CD4+ T-lymphocytes was strongly associated with the progression of illness. The more CD4+ T lymphocytes decline, the higher the risk that severe illness was observed (CDC, 1992). In the early '90s, measures of CD4+ T-lymphocytes were widely used as a determinant of clinical and therapeutic management in United States. In order to reflect the importance of CD4+ T-lymphocyte count in medical

care, and systematize more accurately the HIV-related morbidity, the CDC in the USA decided to revise the HIV infected classification system in 1993 (CDC, 1992).

Based on the clinical conditions associated with HIV infection and CD4+ T-lymphocyte counts, this revised classification system for HIV-infected adolescents and adults divided disease progression into nine categories (Table 1. 3). Three ranges of CD4+ T-lymphocyte counts and three clinical categories are defined in this revision.

Table 1. 3. 1993 Revised Classification System for HIV infection and Expanded AIDS Surveillance Case Definition For Adolescents and Adults. (From CDC report, 1992).

	Clinical categories		
CD4+ T-Lymphocyte Categories	**A Asymptomatic, Acute (primary) HIV or PGL**	**B Symptomatic, not (A) nor (C) conditions**	**C AIDS-indicator Conditions**
1 : ≧ 500 cells/μl	**A1**	**B1**	**C1**
2 : 200 – 499 cells/μl	**A2**	**B2**	**C2**
3 : < 200 cells/μl	**A3**	**B3**	**C3**

[#] Shaded area: Patients with AIDS indicator conditions (category C) and CD4+ T-lymphocyte counts <200 cells/μl (category A3 and B3) were reportable as AIDS in the United States and territories effective January 1, 1993.

[a] PGL, persistent generalized lymphadenopathy.

CD4 positive T-Lymphocyte Categories:

Three categories correspond to CD4+ T-lymphocyte counts per microliter of blood and guide clinical and therapeutic actions in the management of HIV-infected adolescents and adults. It also allows the using of the percentage of CD4 positive T-cells

- Category 1: greater than or equal to 500 cells/μl (greater than or equal to 29%)
- Category 2: 200-499 cells/μl (14-28%)
- Category 3: less than 200 cells/μl (less than 14%)

Clinical Categories:

Category A – consists of one or more of the conditions listed below in an adolescent or adult (greater than or equal to 13 years) with documented HIV infection. Conditions listed in Categories B and C must not have occurred.

- Asymptomatic HIV infection
- Persistent generalized lymphadenopathy (PGL)
- Acute (primary) HIV infection with accompanying illness or history of acute HIV infection

Category B – consists of symptomatic conditions in an HIV-infected adolescent or adult that are not included among conditions listed in clinical category C and that meet at least one of the following criteria: (i) the conditions are attributed to HIV infection or are indicative of a defect in cell-mediated immunity, or (ii) the conditions are considered by physicians to have a clinical course or to require management that is complicated by HIV infection.

Category C – includes the clinical conditions listed in the AIDS surveillance case definition. For classification purposes, once a category C condition has occurred, the person will remain in category C.

1.5.4 Modes of Transmission

A high virus load is observed in blood and body fluids during the phase of acute HIV infection and during the symptomatic period. Any contact with contaminated or infected blood, blood products and body fluids, especially in the acute and symptomatic phases might be expected to present the greatest risk of HIV transmission (reviewed in Levy, 1998). There are three principal modes - sexual, parenteral, and perinatal, which permit the transmission of HIV-1 (Quinn, 1996). Among these three modes, sexual transmission, which includes homosexual and heterosexual transmission, is more important than other two. About 75% of all HIV infections worldwide are acquired in this way (UNAIDS/WHO, 1998).

At the beginning of the HIV pandemic, homosexual transmission was the primary mode in developed countries. However, epidemiological statistics show recently that heterosexual transmission has gradually replaced homosexual as the majority of HIV infections worldwide and was probably already in the majority in some parts of the world. Because of this change, from typical male-to-male sexual transmission to male-to-female transmission, female HIV infections are increasing very quickly, from 25% in 1990 to 45% by 1997 (UNAIDS/WHO, 1998). And, these female HIV infections create another transmitted problem - perinatal transmission

Perinatal transmission might occur by intrauterine infection of fetus, during delivery, exposure of the newborn to maternal blood or postnatally via breast-feeding. The transmission rate from mother to child is about 11 to 60%, which is regionally dependent (Peckham and Gibb, 1995). Perinatal transmission is also influenced by a variety of factors. Generally, the level of infectious virus in the mother at the time of delivery is a critical factor (Scarlatti, 1996).

Parenteral transmission may occur during transfusion with infected blood products, and exposure to infected blood through re-use of needles or syringes among intravenous drug users, or in health care facilities where sterilization of instruments is inadequate (Quinn, 1996). Transmission among IDUs is a serious problem in developed countries, Southeast Asia, and some countries in Latin

America. Recently, the use of sterile injecting equipment has been proved to reduce the rates of HIV-1 transmission among intravenous drug users. This gives some hope for reducing the transmission through IDU (d'Cruz-Grote, 1996; Coates *et al.*, 1996a; Coates *et al.*, 1996b).

Furthermore, to eliminate the transmission through blood transfusion and blood products such as factor VIII and factor IX, a heating procedure is now applied before or after lyophilisation. This has prevented transmission of HIV-1 to haemophiliacs and other users of blood products (reviewed in Levy, 1998).

1.5.5 AIDS Epidemic

AIDS has now risen to the level of global pandemic proportions and the number of HIV infections is growing frighteningly fast, especially in Africa and South East Asia. Statistics from the annual report of the Global HIV/AIDS Joint Programme indicates that over 32.4 million adult and 1.2 million children are living with HIV/AIDS at the end of 1999, and that about 16 million people around the world had already died with AIDS (UNAIDS/WHO, 1999). An estimated 5.6 million adult and 570,000 children were newly infected in 1999.

1.5.5.1 Regional Epidemics

According to various factors, such as the time that the epidemic started, the density and number of population, and cultural effects, the HIV epidemics in different geographic regions possess their own dynamics. UNAIDS and WHO reporters divide the global HIV infected populations into 8 groups: Market Economies, Caribbean, Latin America, North Africa and Middle East, sub-Saharan Africa, Eastern Europe and Central Asia, East Asia and Pacific, South and South-East Asia. The regional statistics and features are summarized in (Table 1. 5).

Table 1. 4. Global summary of the HIV/AIDS epidemic, December 1999

(Modified from the Report on the global HIV/AIDS epidemic update: December 1999)

Number of people living with HIV/AIDS	Total	33.6 million
	Adults	32.4 million
	Women	14.8 million
	Children < 15 yrs	1.2 million
People newly infected with HIV in 1998	Total	5.6 million
	Adults	5 million
	Women	2.3 million
	Children < 15 yrs	570,000
AIDS death in 1998	Total	2.6 million
	Adults	2.1 million
	Women	1.1 million
	Children < 15 yrs	470,000
Total number of AIDS deaths since the beginning of the epidemic	Total	16.3 million
	Adults	12.7 million
	Women	6.2 million
	Children < 15 yrs	3.6 million
Total number of AIDS orphans* since the beginning of the epidemic		8.2 million

*Defined as children who lost their mother or both parents to AIDS when they were under the age of 15.

Table 1.5. Regional HIV/AIDS statistics and features, December 1999 ***(Copied from the Report on the Global HIV/AIDS Surveillance - December 1999)***

Region	Epidemic started	Adults & children living with HIV/AIDS	Adults & children newly infected with HIV	Adult prevalence rate (*)	Percent of HIV-positive adults who are women	Main mode (#) of transmission for adults living with HIV/AIDS
Sub-Saharan Africa	Late '70s – early '80s	23.3 million	3.8 million	8.0%	55%	Hetero
North Africa & Middle East	Late '80s	220,000	19,000	0.13%	20%	IDU, Hetero
South & South-East Asia	Late '80s	6 million	1.3 million	0.69%	30%	Heterp
East Asia & Pacific	Late '80s	530,000	120,000	0.068%	15%	IDU, Hetero, MSM
Latin America	Late '70s – early '80s	1.3 million	150,000	0.57%	20%	MSM, IDU, Hetero
Caribbean	Late '70s – early '80s	360,000	57,000	1.96%	35%	Hetero, MSM
Eastern Europe & Central Asia	Early '90s	360,000	95,000	0.14%	20%	IDU, MSM
Western Europe	Late '70s – early '80s	520,000	30,000	0.25%	20%	MSM, IDU
North America	Late '70s – early '80s	920,000	44,000	0.56%	20%	MSM, IDU, Hetero
Australia & New Zealand	Late '70s – early '80s	12,000	500	0.1%	10%	MSM, IDU
Total		33.6 million	5.6 million	1.1%	46%	

*The proportion of adults (15 to 49 years of age) living with HIV/AIDS in 1999, using 1998 population numbers

MSM (Sexual transmission among men who have sex with men), IDU (Transmission through injecting drug use), Hetero (heterosexual transmission).

Sub-Saharan Africa

The biggest HIV-infected population is located south of the Saharan desert. Around 21 million adults and children were living with HIV/AIDS in this area by the end of 1997, which is equal to two-thirds of the HIV global infected population. The mode of transmission in this region is usually by heterosexual intercourse. In comparison with other areas in which the major transmission route is by means of male-with-male sexual activity or by blood contamination in intravenous drug injection, 80% of the global HIV-positive female population live in sub-Saharan Africa indicating their high risk of infection in this area. Moreover, African women have more children on average than do other women worldwide. Infected mothers may pass the virus on to their children during pregnancy or during childbirth or through breast-feeding. Therefore, 87% of the global population of HIV-positive children are also in Africa. Though zidovudine (AZT) and some of the newer antiretroviral drugs can reduce this vertical transmission, the high price of these drugs means that they are less available in these indigent countries so that the prevalence of HIV in this area is accordingly likely to rise even faster than before (reviewed in UNAIDS/WHI, 1998; UNAIDS/WHO, 1999).

Asia

The HIV pandemic started relatively later in Asia than in the rest of the world. However, the prevalence increased sharply from the early '90s. By the end of 1997, an estimated 6 million people were living with HIV/AIDS in South East Asia, and 1.3 million were newly infected during 1997 (UNAIDS/WHO, 1998).

HIV was first introduced among drug injectors around the "Golden Triangle" area. Subsequently, female sex workers were found to have been infected following commercial sex with these drug injectors. Although highly variable by region, HIV then spread rapidly through commercial heterosexual intercourse, especially in Vietnam, Thailand and India. For the more developed countries such as Japan, South Korea, Taiwan, Singapore and Hong Kong, the HIV epidemics have not grown as rapidly as in South East Asia, but it is believed that the number of HIV infected subjects is increasing slowly and steadily (Kaldor *et at.,* 1994) .

Established Market Economies, Latin America, Caribbean

HIV epidemics commenced at the beginning of the '80s in Latin America, the Caribbean and in industrial countries, including Western Europe, North America, Australia and New Zealand. Up to 1997, there were about 3 million infected individuals living in these regions. The epidemic was initially distributed among homosexual/bisexual men, and drug injectors. Infections among women increased after 1995, due to heterosexual contact with partners who were in high-risk groups. Fortunately, because of good education and the use of antiretroviral drugs, the infection rate in these areas is falling slightly (d'Cruz-Grote, 1996; UNAIDS/WHO, 1998).

North Africa, Eastern Europe, Middle East

The epidemiological data is not well established in these regions. However it is unlikely that these areas will escape the pandemic of HIV/AIDS. An estimated 360,000 people were infected with HIV by the end of 1997 in these regions, and the rate of infection has increased sharply since 1995, especially among big cities.

The pattern of HIV/AIDS spread and pathogenicity is clearer in a variety of fields than before. However, there are still around 16,000 new HIV infections worldwide every day. To have a clear understanding of HIV/AIDS pathogenicity is a priority, so that the rate of spread of the infection is slowed down and so that more practical treatment may be developed (UNAIDS/WHO, 1998).

1.6 Cytopathic Properties of HIV-1

1.6.1 General Immune Response

During acute HIV infection, very high levels of infectious virus can be detected in the peripheral blood. Large numbers of cells, mainly CD4+ cells, are infected at this stage. Generally, within weeks after acute infection, viremia is markedly reduced, the number of CD4+ cells is decreased, and in contrast, the number of CD8+ cells is increased in blood (Pantaleo *et al.,* 1994; Feinberg, 1996; Levy, 1998).

A few months after primary virus infection, virus replication is observed to stay at a low level, although still continuing. The disease at this stage is asymptomatic. The CD4+ cell numbers at this stage usually rise to near-normal levels, followed by a steadily decline until the symptomatic stage, and the number of CD8+ cells remains slightly raised (Levy, 1998).

When the individual develops symptoms, the number of CD4+ cells has usually dropped below 200 cells/μl. The virus in the blood and lymphoid tissues rises again to high levels, and the response of antiviral CD8+ cells is also decreased. Eventually in the absence of treatment the whole immune response fails. Interestingly, investigators have found that most T cells that die during HIV infection do not appear to be infected with HIV (Thompson, 1993). This dramatic depletion of CD4+ and CD8+ cells is thought to be responsible for the severe immunosuppresssion and the development of opportunistic infections and neoplasms (Pantaleo *et al.,* 1993b). Although the exact mechanisms of the loss of immune cells remain unclear at present, the possible factors involved might be direct cell destruction by HIV-1, or by viral proteins, or as the result of secondary effects of the immune system disorder (reviewed in Levy, 1998).

1.6.2 Mechanisms of Cytotoxicity

1.6.2.1 Syncytium Formation

The first sign of HIV-1 infection of PBMCs in culture is the formation of multinucleated cells, also known as syncytium formation. This kind of cell-to-cell fusion results when uninfected CD4+ cells fuse with HIV-1-infected cells (Lifson, 1993; reviewed in Levy, 1998). It is temperature dependent, and does not require cellular DNA, RNA or protein synthesis (Frey *et al.*, 1995). Although the mechanism of syncytium formation is still a mystery, the HIV-1 envelope glycoproteins and cell surface CD4 molecule are believed to be involved in this process (Frey *et al.*, 1995; Dragic *et al.*, 1995). It is known that HIV-1 gp120 has a high affinity for the cell surface CD4 molecule. During virion assembly, these envelope proteins are inserted into the host cell membrane, which then have an opportunity to bind to the CD4 molecules on the surface of uninfected cells, hence leading to syncytium formation (Dragic *et al.*, 1995). These fused cells died in culture shortly after they were formed (Lifson, 1993). This observation suggests that cell-to-cell fusion leads to viral cytopathicity and that cell death may be one mechanism to account for the depletion of CD4+ cells (Levy, 1998). Moreover, this cell-to-cell fusion provides an even more effective route for HIV-1 diffusion. It has been estimated that cell-to-cell transmission might be 100 times more efficient than infection by free virus particles (Sato *et al.*, 1992).

According to their ability to induce cell fusion in established T-cell lines, isolates of HIV-1 have been divided into two major groups, syncytium-inducing (SI) strains that replicate rapidly and cause syncytium formation, and non-syncitium-inducing (NSI) strains that replicate slowly and do not form syncytia (Fenyo *et al.*, 1988). The cellular target of viruses with the SI phenotype is lymphocytes, and isolates of SI strains are commonly associated with patients who have advanced disease. The cellular target of NSI phenotypic viruses is preferentially monocyte-derived macrophages, and NSI species are generally isolated from patients who are asymptomatic (reviewed in Levy, 1998).

1.6.2.2 Toxicity of HIV-1 and Viral Proteins

Excessive budding of virus particles from infected cells could also lead to cell death. When high levels of HIV-1 virions are produced, consistent budding from the cell surface could weaken the integrity of the cell membrane resulting in membrane discontinuities and pores. Voss *et al.* suggested that cells are unable to control the influx of monocovalent and divalent cations while virus production is in progress (Voss *et al.,* 1996). The loss in control of the intracellular ionic strength would change the electrical potential of the cell, leading to the impairment of cellular functions and eventual lysis of the cells (Voss *et al.,* 1996).

Accumulation of viral cDNA in the cytoplasm of infected cells in culture is also likely to lead to cell death (Levy, 1998). In the study of Pang *et al.,* neurologic damage has been found to be associated with large amounts of unintegrated HIV-1 DNA in the brain cells of AIDS dementia patients (Pang *et al.,* 1990). Tang *et al.* further confirmed that the persistent production of unintegrated cDNA in HIV-1-infected T cells appeared to block cell replication leading to cell death (Tang *et al.,* 1992). These observations suggest that high levels of intracellular DNA could be toxic to the cell and could contribute to the initial cell killing observed in the early stages of infection (Levy, 1998).

Other investigations have demonstrated that several HIV-1 viral proteins are cytotoxic for CD4+ cells. For example, HIV-1 envelope gp120 and gp41 proteins were reported to be toxic to cells, most probably through altering the membrane permeability (Miller *et al.,* 1993). Also, gp120 and gp41 presumably played important roles in cell fusion that accompanied cell death. Lamb & Pinto showed that the auxiliary viral proteins Vpu and Vpr could affect cell viability through their ion channel activities (Lamb & Pinto, 1997). Moreover, the presence of excessive Nef and Tat proteins in culture systems is found to cause the death of neuronal cells (Jones *et al.,* 1998).

1.6.2.3 Effects of Host Immune Responses

The host immune system plays a vital role in arresting or eliminating the infectious agent. Since in HIV infection, it is cells of the immune system which are infected, the destruction of virus-infected cells either by cell-mediated cytotoxicity or humoral immunity is thought to be the major mechanism leading to loss of immune cells, and resulting in diseases.

Cell-Mediated Cytotoxicitiy

The most appropriate immunologic defense against certain viral infections is cell-mediated cytotoxicity, which is primarily generated by cytotoxic T lymphocytes (CTLs). This response is major histocompatibility complex (MHC) class-I-restricted, antigen specific, and requires cell-to-cell contact. CTLs, also known as CD8+ lymphocytes, function to recognise viral antigen processed endogenously in infected cells and presented on their surface as a binary complex of a peptide fragment and the human leukocyte antigen (HLA), an human equivalent of the MHC molecule. Following recognition, CTLs are activated and finally kill the infected cells. Besides this specific CTL activity, other studies demonstrated that cytotoxic CD8+ cells recovered from the blood of HIV-infected individuals may mistakenly kill uninfected CD4+ cells that were coated with soluble gp120 produced elsewhere, causing the lysis of these CD4+ cells (Wilson *et al.*, 1997).

Although HIV-induced CTL activity is thought to be one of the mechanisms leading the depletion of immune cells, other studies have proposed a protective role for CTLs in HIV-1 infection (Kazi *et al.*, 1996; Wolinsky *et al.*, 1996). Wolinsky *et al.* demonstrated that there was an inverse relationship between progression to disease and the humoral or CTL immune response (Wolinsky *et al.*, 1996). They found an increased CTL activity in those individuals showing a slower rate of disease progression, thus suggesting that CTL activity may protect the host (Wolinsky *et al.*, 1996). Moreover, the anti-HIV activity of these CD8+ lymphocytes might present clinically as a diffuse infiltrative lymphocytosis syndrome (Kazi *et al.*, 1996). This syndrome is characterised by a CD8+ cell infiltration, which is primarily found in

salivary glands, lungs, gastrointestinal tract, and kidney. Patients with this syndrome usually have a delay in CD4+ T-cell loss and a longer survival period (Kazi *et al.*, 1996).

Other than CD8+ cells, some CD4+ cells are also capable of CTL activity directed either at infected or uninfected CD4+ cells, or at cells expressing HIV peptides in association with the MHC class II molecule (Miskovsky *et al.*, 1994). Similar to cytotoxic CD8+ cells, these CD4+ lymphocytes with CTL activity may help to eliminate HIV-1 through cell-mediated responses, but could also have a detrimental effect on uninfected cells (Miskovsky *et al.*, 1994). The rapid lysis observed between CD4+ cells and infected target cells in culture appears to be a result of CTL activity (Miskovsky *et al.*, 1994), and may be related to cell-to-cell contact and syncytium formation (Heinkelein *et al.*, 1995). However, no HLA-class-II restricted CTLs (CD4) has been detected in fresh tissues of infected patients, and this CD4 CTL activity has been detected only following repeated stimulation *in vitro*. Thus, their role in the HIV-1 immune response requires further investigation.

Humoral Immune Rresponse

Antibody-dependent cellular cytotoxicity (ADCC) responses, which are mediated by the antibodies to HIV-1 envelope proteins, may also contribute to the depletion of immune cells (Skowron *et al.*, 1997). Generally, the antibody-antigen-coated cells are recognised by NK cells or by monocytes or macrophages bearing Fc receptors, and then killed by a cytolytic mechanism (reviewed in Levy, 1998). However Weinhold *et al.* demonstrated that uninfected cells might be destroyed by non-specific ADCC responses in culture (Weinhold *et al.*, 1989). The gp120 is presumed to be released from the budding virions or following the lysis of infected cells. This freely circulating gp120 could bind to the CD4 receptor on the surface of uninfected CD4+ cells, result in an appearance of infection and therefore becoming a target for lysis by ADCC responses (Tyler *et al.*, 1989; Weinhold *et al.*, 1989).

Effects of Complement

Complement can play a role in the antiviral response by enhancing complement-mediated lysis of virions or by destroying virus and virus-infected cells through binding and activation by the classical or alternative pathway (Susal *et al.*, 1994). Moreover, complement could indirectly lyse the CD4+ cells carrying envelope proteins, causing the loss of CD4+ cells (Marschang *et al.*, 1997).

Apoptosis

Apoptosis, a kind of programmed cell death is a morphologically distinct form of cell loss and occurs through the activation of a complex cell-intrinsic suicide program. It plays a major role during development, homeostasis, and in many diseases including cancer, AIDS, and neurodegenerative disorders (Steller, 1995) and has been proposed as one of the mechanisms involved in CD4+ T cell loss during HIV-1 infection (Pantaleo & Fauci, 1995; Tian *et al.*, 1996). Apoptosis is usually associated with the activation of a Ca^{2+}-dependent endogenous endonuclease that degrades the chromosomal DNA into very small nucleotide units that are then membrane-bound, followed by rapid phagocytosis and digestion by macrophages or neighboring cells (Steller, 1995; Sasaki *et al.*, 1996).

There is no clear understanding as yet on how apoptosis is linked to HIV pathogenesis. Some investigators have suggested HIV-1 viral proteins and superantigens might contributed to the induction of apoptosis (Pantaleo & Fauci, 1995; Tian *et al.*, 1996). Evidence has emerged that cross-linking of HIV gp120 bound to CD4+ lymphocytes followed by T-cell activation has caused apoptosis of these cells (Pantaleo & Fauci, 1995). Also, HIV-1 Vpr could cause apoptosis of CD4+ cells by blocking virus replication at the G2 stage of the cell cycle (Kolesnitchenko *et al.*, 1995). Moreover, HIV-1 Tat protein has been shown to induce apoptosis in normal, uninfected CD4+ cells through the activation of cyclin-dependent kinases (Li *et al.*, 1995).

Superantigens can play a role in up-regulating cell replication and inducing cell

activation associated with apoptosis (reviewed in Levy, 1998). In general, antigens must bind in the groove of the MHC class II molecule interacting with both the variable elements of the α and β subunits (V_α and V_β) of the T cell receptor (TCR). Superantigens differ from typical antigens in that they are less specific and are required only to bind to the V_β subunit of the TCR. However they can stimulate and activate a much larger proportion of T cells than conventional antigens. These activated T cells may be deleted, rendered anergic or made more susceptible to viral infection (Pantaleo *et al.*, 1993b). It has been postulated that superantigens are implicated in HIV-mediated immunosuppression. The actual mechanisms and possible effects of superantigens involved in HIV-1 pathogenesis are still unknown. Recently, a study found that a portion of HIV-1 gp120 or immune complexes with this glycoprotein could serve as superantigen inducing some of the B-cell responses associated with HIV infection (Karray & Zouali, 1997). It is likely that superantigens are not directly responsible for the loss of immune cells, but may be indirectly involved by activation of large number of T cells, resulting in enhanced HIV-1 infection, replication and subsequent cytopathogenicity (Cossarizza *et al.*, 1995).

1.6.3 General Pathology of Organ Infections

It is known that severe depletion of CD4 lymphocytes is followed by a severe deficit in immunological responses. At the late stages of HIV infection, patients become extremely sensitive to infectious agents and may also develop certain neoplasms. Most of the infections associated with AIDS are caused by opportunistic organisms including viruses, bacteria, fungi and protozoa. Evidence from clinical observation confirm that HIV-1 is a systemic disease affecting multiple organs or systems of the body. Neoplasms or opportunistic infections in organs or systems are responsible for the death of untreated HIV-infected individuals (Feinberg, 1996).

The following sections summarize the general pathological findings and the possible mechanisms of systemic disorders in lymphoid organs, central nervous system, respiratory system, and gastrointestinal system. These four systems are the major targets of HIV-1 infection.

1.6.3.1 Lymphoid Organs

Lymphoid organs are the primary anatomic sites for HIV replication and propagation following acute HIV-1 infection (Pantaleo *et al.*, 1993c; Pantaleo *et al.*, 1994). At the early stage of HIV-1 infection, some patients are unaware of any constitutional changes and show no symptoms, others might develop flu-like acute illnesses and probably a syndrome of persistent generalized lymphadenopathy (PGL) (Spira *et al.*, 1989). PGL is an early and relatively mild manifestation of HIV-1 infection and characterised by lymph nodes of one-centimeter or more in diameter in two or more non-contiguous extrainguinal sites that cannot be explained by any other infection or condition. The nodes are symmetrical, mobile and non-tender and generally occur in cervical and axillary sites (Pantaleo *et al.*, 1994).

Initially, these reactive lymph nodes show hyperplasia and high levels of virus replication (Pantaleo *et al.*, 1994). These nodes enlarge, with particular increase in the secondary germinal centers (or follicles) filled with activated polyconal B cells (Burke *et al.*, 1994). Moreover, the paracortical areas increase in size with the expanding number of CD8+ lymphocytes that might reflect the enhanced production of β-chemokines by resident macrophages (Tedla *et al.*, 1996). Also, many infected CD4+ cells are detected in the follicular mantle and paracortical regions in this acute period (Tedla *et al.*, 1996).

Subsequently in the asymptomatic period, large numbers of virus-infected CD4+ cells are observed to reside in lymph nodes, particularly in the mantle regions. Interestingly, most of these CD4+ cells are infected latently (Embretson *et al.*, 1993b; Rosok *et al.*, 1997). Moreover, histologic and electron microscopic examinations have indicated that viral particles can be visualized in the villous processes of follicular dendritic cells (FDC) at this stage (Pantaleo *et al.*, 1993a). FDCs are localised in the germinal centres and thought to be the largest reservoir of virus in lymphoid tissue during this transient period (Pantaleo *et al.*, 1993c; Haase *et al.*, 1996).

With advancement to symptomatic disease, the lymph nodes atrophy and

gradually lose their normal architecture owing to the slow but persistent destruction of CD4+ cells, deterioration of the stromal elements and release of virus from intercellular trappings in the FDCs or from infected lymphoid cells producing virus. As the FDC network undergoes as destruction, immune function falters, with consequent return to high levels of virus replication in peripheral blood and lymphoid tissues (Pantaleo *et al.*, 1994). In the end stages of disease or AIDS, the viral load in lymph nodes is decreased and the viral expression in the peripheral blood is increased to a high level. The findings suggest a breakdown in the FDC network and release of virus into the blood. Eventually, Lymph nodes show typical atrophy, with marked depletion of CD4+ cells and severe destruction of FDCs (Pantaleo *et al.*, 1994). The loss of these cells may be due to the toxicity of virus and its proteins, the production of cytokines by cells in responding to increase virus replication, or cytotoxic activity of active CTLs (Nuovo *et al.*, 1994; Pantaleo *et al.*, 1995). Finally, these immune organs are destroyed and lose their function.

Through the AIDS stage of HIV-1 infection, neoplasms and opportunistic infections may occur in lymphoid organs. Neoplasms such as malignant lymphomas and Kaposi's sarcoma (KS) arising in AIDS resemble those complicating non-AIDS immunosuppressive disorders in frequency and behaviour and they appear to occur in unusual sites (Schulz *et al.*, 1996; Goedert *et al.*, 1998). Malignant lymphomas occur in patients with AIDS as they do in the general population, but with a significantly increased incidence (Knowles *et al.*, 1988). High-grade non-Hodgkin's lymphoma (NHL) is a neoplasm frequently associated with AIDS, and is most often of B-cell type (Knowles *et al.*, 1988). Up to 90% of AIDS associated NHL occurs in atypical extranodal locations including the lymphoid tissues associated with bone marrow, gastointestinal tract and liver. Kaposi's sarcoma is a vascular tumor, which is the most common neoplasm in patients with AIDS (Beral *et al.*, 1991). Originally, KS was considered a rare, slowly progressive tumour of elderly Mediterranean men. In the early 1980s, KS was observed in AIDS patients and more commonly associated with HIV-1 infection in homosexual or bisexual men than in other risk groups (Beral *et al.*, 1992). This variant, unlike the classic KS, is aggressive and progresses rapidly. This AIDS-associated KS disseminates widely and includes the

viscera and lymph nodes as well as skin (Beral *et al.*, 1992), but does not generally involve the central nervous system.

Lymph nodes are commonly sites for opportunistic infections in AIDS, usually resulting in lymphadenopathy. Generally, the infections are multiple and are sometimes found in association with neoplasms. Atypical mycobacterial infection including those due to *Mycobacteria avium-intracellulare* (MAI) and other closely related organisms, are frequently seen in AIDS patients (Schulz *et al.*, 1996). With MAI, the normal cortex and medulla structure of lymph nodes is almost completely replaced by a diffuse and heavy infiltrate of macrophages containing numerous bacteria (Levy, 1998).

The spleen is another lymphoid organ, and the HIV related changes reflect the pathology elsewhere in the lymphoid system. With HIV-1 infection, it shows profound atrophy of T- and B-cell zones, with total loss of germinal centres. Lymphocyte depletion is the major abnormality seen in spleen with HIV-1-infected individuals (Pantaleo *et al.*, 1994; Levy, 1998).

1.6.3.2 Central Nervous System

Outwith the immune system, the other major target of HIV-1 is the CNS. Approximately 80% of AIDS patients experience neurological or psychiatric symptoms. The symptoms of HIV-1 CNS involvement may appear gradually and become more severe with disease progression. Clinical manifestations of CNS involvement in HIV-1 infection include headaches, confusion, memory loss, impairment of movement, emotional/mood swing, and behavioural difficulties eventually progressing to dementia in approximately 10 % of patients with AIDS (Gray *et al.*, 1996; Price, 1996; Bell, 1998). These symptoms together with a progressive cognitive decline have collectively been termed AIDS related dementia (ARD) and classified as an AIDS defining illness (Price *et al.*, 1996). Common problems occurring in the CNS include HIV-1 specific neuropathology, opportunistic infections, and neoplasms (Gray *et al.*, 1996; Price *et al.*, 1996; Bell,

1998). Approximately 10 % of untreated AIDS patient develop dementia.

HIV-encephalitis (HIVE) is a direct effect of HIV-1 infection of the CNS and occurs in AIDS patients. Many patients with HIVE often suffer physiological and psychological problems, and most will develop dementia. The definitive characteristics of HIVE are the presence of multi-nucleated giant cells (MGCs) and/or immunocytochemically detected HIV antigens in brain parenchymal cells. MGCs are generally found in the central white matter and deep grey matter, and are thought to be formed by fusing HIV-1 infected and possibly uninfected cells. The presence of HIV-1 antigens or nucleic acid must be demonstrate by immunohistochemistry staining or nucleic-acid-based detecting techniques before HIV-1 encephalitis can be definitively diagnosed (reviewed in Bell, 1998). Microglial nodules are focally concentrated collections of microglial cells, macrophages and lymphocytes and they may be observed in both grey and white matter (reivewed in Bell, 1998). MGCs may be absent in HIVE cases.

Another HIV-related abnormality in the CNS is HIV leucoencephalopathy (HIVL), which is defined as white matter damage associated with diffuse myelin loss and reactive astrocytosis, but with little or no inflammatory infiltrate. The diagnosis between HIVE and HIVL is not entirely straight forward because they both present MGCs and immunocytochemical evidence of the presence of HIV antigens (reviewed in Bell, 1998). The histological findings should be carefully reviewed since different pathogenic mechanisms may be involved.

Opportunistic infections occur frequently in immuno-compromised hosts when their immune systems are failing. Most of the pathogens responsible for the opportunistic infections are ubiquitous organisms to which commonly exposure occurs. Cytomegalovirus (CMV) and *Toxoplasma gondii* are the pathogens that are found commonly in the CNS in AIDS patients. Sometimes, progressive multifocal leukoencephalopathy (PML) and fungal infectious also occur. They may affect the CNS in isolation or associated with HIVE (reviewed in Bell, 1998).

The most commonly found neoplasms in CNS of AIDS patients are lymphomas, especially the high grade B-cell, non-Hogdkin's lymphomas (Bell, 1998). Primary CNS lymphoma is more common than secondary, and is most frequently found in the periventricular deep grey matter, brain stem or cerebellum. Generally, neoplasms occur during the late stages of illness and accompany other late effects of HIV-1 infection.

Other CNS pathology, including gliosis, astrocytosis, inflammatory infiltrates and vascular abnormalities, is frequently present and may be accompanied by hepatic encephalopathy in patients who co-infected with hepatitis B or C (Price *et al.*, 1996; Bell, 1998).

Although there is frequent involvement of the CNS in HIV-1 infection, the mode and timing of viral entry and the neuropathogenic mechanisms of HIV-1 are incompletely understood at present. The two most widely accepted theories are that viral entry occurs early in infection with the virus remaining latent during pre-AIDS, then becoming productive when systemic immunosuppression is evident (Saito *et al.*, 1994; Gray *et al.*, 1996), or that major viral entry to the brain is a late event in HIV-1 infection (Bell *et al.*, 1993; Donaldson *et al.*, 1994a). Using PCR analysis, some studies have detected HIV-1 provirus DNA in the brains of some asymptomatic HIV-positive patients (Sinclair *et al.*, 1992; Sinclair *et al.*, 1994). This gave support to the idea that viral entry might occur early either during the acute seroconversion reaction or at the time of subclinical infection (Nottet and Gendelman, 1995).

Several mechanisms have been proposed for the entry of HIV-1 to brain tissue. The first is the "Trojan horse" hypothesis, which suggested that HIV-1 infected monocytes act as a "Trojan horse" to carry virus across the tight interendothelial junctions of the parenchyma blood brain barrier (Peluso *et al.*, 1985). Later, Moses *et al.* demonstrated that brain microvascular endothelial cells (BMVECs) are capable of becoming infected with lymphotropic HIV-1 (Moses *et al.*, 1993). This result suggested that direct infection of the BMVECs might also be a route for the entry of HIV-1. Recently, Falangola *et al.* and Bagasra *et al.* reported productive infection of

the choroid plexus using immunohistochemistry and the in situ PCR technique (Falangola *et al.*, 1995; Bagasra *et al.*, 1996). The choroid plexus capillaries have gap junctions and lie outside the brain blood barrier (BBB). It has been implicated as the initial site of entry of infective agents due to its open capillary architecture (Falangola *et al.*, 1995). Many HIV-1 positive cells were identified as monocytes and macrophages in the choroid plexus stroma (Falangola *et al.*, 1995). It has been hypothesised that HIV-1 infected cells in the choroid plexus could move from the blood to the choroid plexus and into circulating CSF and possibly access brain parenchyma, causing infection (Falangola *et al.*, 1995; Bagasra *et al.*, 1996). More recently, Nottet *et al.* found that HIV-infected monocytes might induce the expression of adhesion molecules on the BMVECs that allow binding and infiltration of HIV-infected monocytes into brain (Nottet *et al.*, 1995). Their investigation demonstrated that active HIV-infected monocytes express the cytokines, TNF-α and IL-1β, in a culture system. These cytokines in vivo could induce microvascular endothelial expression of vascular cell adhesion molecule 1 (VCAM-1) and E-selectin, which mediate transendothelial migration of HIV-infected cells, causing infection of brain microglial cells (reivewed in Nottet & Gendelman, 1995).

Many cells have been proposed to harbour HIV-1 in CNS. Most of them belong to the monocyte/macrophage lineage, such as brain macrophages, microglia and MGCs. However infection of cells of neuroectodermal origin, including neurons, astrocytes and oligodendrocytes is rarely reported (reviewed in Bell, 1998), although astrocytes may support a form of non-productive HIV infection. Therefore HIV-1 is likely to exert its damaging effect indirectly in tissue of the CNS (Epstein & Gendleman, 1993). The most favoured theory is that infected cells release toxic cellular factors leading to tissue damage and/or apoptosis. It is believed that after infection with HIV-1 or exposure to viral envelope proteins, the microglial and brain macrophages could be induced to produce certain cytokines, such as TNF-α, IL-1 and IL-6, which are toxic to the brain tissue (reviewed in Levy, 1998). IL-1 and IL-6 have been reported to be cytotoxic, induce proliferation of astrocytes and cause fever and inflammation (reviewed in Levy, 1998). TNF-α has also been reported to contribute to neural damage by calcium influx (Soliven & Albert, 1992), stimulation

of astrocytosis, induction of myelin damage and destruction of oligodendrocytes (Nottet & Gendelman, 1995). Moreover, the interaction between envelope protein gp120 and glutamate via the N-methyl-D-aspartate (NMDA) receptor may mediate excessive calcium influx into the neurons with subsequent neuron injury (Pittaluga *et al.*, 1996). The neuropathogenesis is complex, with the effects of cytokines, as well as cytotoxic HIV-1 viral proteins thought to be responsible for neural dysfunction.

1.6.3.3 Respiratory System

Pulmonary complications appear in the late stage of HIV-1 infection. Over 60 percent of AIDS patients develop pneumonia. Generally, the respiratory failure is a major cause of death of AIDS patients, manifested by severe refractory hypoxia (Miller, 1996; Zuckerman *et al.*, 1996).

Most of the cases with pneumonia are infected with *pneumocystis carinii,* which presents as *Pneumocystis carinii* pneumonia (PCP), an AIDS defining illness (Marchevsky *et al.*, 1985; Huang & Stansell, 1996; Miller, 1996). Other respiratory disorders including infections due to cytomegalovirus (CMV), mycobacterium, both atypical and *M. tuberculosis*, pneumococci, cryptococci and other fungi may also be present in AIDS patients. The biggest problem in treating these infections is that in 5-10 % of cases, pneumonia is mixed with coexisting infections by two or more of these organisms. This often leads to treatment failures when only one of the infectious agents has been identified. Other than infections, neoplasms, such as KS, could also occur in respiratory system (reviewed in Miller, 1996).

Pneumocystis carinii is a unicellular, opportunistic organism responsible for atypical pneumonia. PCP may occur in conditions other than AIDS in patients suffering from malignant lymphomas and leukaemias as well as those on imunosuppressive therapy or on long-term steroid or cytotoxic therapy. With the appearance of AIDS the incidence of PCP increased dramatically (Masur *et al.*, 1989). Within the lung the pneumocysts are found primarily in the alveoli.

Replicating pneumocysts appear to increase the permeability of the alveolar capillary membrane leading to intra-alveolar oedema (Marchevsky *et al.*, 1985; Harcourt-Webster, 1993).

CMV peumonitis is the second most common respiratory infection in AIDS (Harcourt-Webster, 1993; Miller, 1996). The characteristic feature of CMV infection is CMV inclusion bodies within the nucleus of the infected cells. The increased protein synthesis leads to enlargement of the cell and the formation of viral particles. Generally, CMV inclusion bodies may be observed in both alveolar and endothelial cells in the lung. It is often a bystander infection rather than a tissure-damaging pathogen in the respiratory system in AIDS patients (Millar, 1996).

MAI are the most common organisms in respiratory system in AIDS. Within AIDS, it occurs more frequently in extra-pulmonary sites (Maguire *et al.*, 1987). HIV-1 can be isolated from the respiratory system even from occasional asymptomatic individuals however none of the abnormalities occurring in lung are directly linked to the infection of HIV-1 (Clarke *et al.*, 1995). Studies have demonstrated that the risk of developing respiratory disorders in HIV-1 infected patients is much higher than in the general population (Masur *et al.*, 1989; Harcourt-Webster, 1993). Whether HIV-1 is involved in the pathogenesis of respiratory system disorders, or these respiratory diseases happen only because of the suppression of the host immune system requires further investigation.

1.6.3.4 Gastrointestinal System

The intestinal tract is both a digestive and an immunological organ. Bowel symptoms have been reported in association with acute HIV-1 infection (Sharpstone & Gazzard, 1996). The subsequent chronic malabsorption, malnutrition, and diarrhoea occurring several years later have generally been attributed to opportunistic infections in the bowel as a result of immune deficiency (DuPont & Marshall, 1995; Sharpstone & Gazzard, 1996).

Malnutrition is considered to be present in a patient with a loss of more than 10% of normal body weight and with greater protein loss than is observed in other cases of starvation. Malnutrition in HIV-1 infection can result from anorexia or nutrient malabsorption secondary to intestinal damage and inflammation (DuPont & Marshall, 1995; Sharpstone & Gazzard, 1996).

Chronic diarrhoea and weight loss are the most common complications in the gastrointestinal (GI) system associated with HIV-1 infection and AIDS. Weight loss may occur with diarrhoea or in association with other HIV-associated infections and disorders (DuPont & Marshall, 1995), such as CMV infection, *Cryptosporidiosis*, MAI, and microsporidia infection. Usually, the gastrointestinal tract shows only subtle histologic changes during HIV-1 infection unless opportunistic infections are present (Harcourt-Webster, 1993).

CMV infection is commonly seen in AIDS patients with symptoms associated with malabsorption or protein-losing enteropathy in GI system. Large number of inclusion bodies may be found throughout the colon, and particular in the ascending colon. Generally, CMV infection of the intestine occurs with minimal non-specific chronic inflammation in the lamina propria; however when the infection becomes severe and highly active, widespread inflammation accompanied by necrosis, haemorrhage and bowel rupture may be seen (reviewed in Harcourt-Webster, 1993).

Infection with *Cryptosporidiosis* is rare in the immunocompetent, and manifests as a self-limiting, non-toxic, mild watery diarrhoea, also known as "traveller's diarrhoea" (DuPont & Marshall, 1995). However the incidence of this kind of infection is greatly increased in HIV-positive patients. *Cryptosporidiosis* is a coccidian protozoon prevalent in bovine animals but can be transmitted to human through contaminated food or water. With immunosuppression, infection with *Cryptosporidiosis* in the intestine may cause a diarrhoea that is severe, cholera-like, non-bloody and very watery and accompanied by anorexia, vomiting, abdominal pain and cramps with fever (DuPont & Marshall, 1995).

Other opportunistic infections including MAI and microsporidia may occur infrequently. KS and malignant lymphoma, especially non-Hodgkin's lymphoma, may also involve the gastrointestinal tract. The small intestine is thought to be the most frequent of all the extranodal sites for lymphoma (Harcourt-Webster, 1993).

It is known that the major protection in the intestinal system is generated by the mucosal immune system (MIS) (DuPont & Marshall, 1995). The MIS consists of focal aggregates of cells and tissue from the epithelium to the lamina propria and includes T cells, B cells, mast cells, and macrophages. Among these cells, CD4+ T cells and macrophages are susceptible to infection by HIV-1. Although no direct correlation was found between HIV-1 infection and gastrointestinal symptoms, studies investigating the cause of gastrointestinal disturbances have demonstrated the presence of HIV-1 in the bowel mucosa of HIV-1-positive patients with intestinal disorders, including asymptomatic individuals (Kotler *et al.*, 1991; Levy, 1998). Moreover, in some cases, HIV-1 was the only pathogen identified in bowel (reviewed in Levy, 1998). These unique bowel-derived HIV-1 strains, which are often macrophage-tropic, can be distinguished from strains recovered from the blood of the same individual (Barnett *et al.*, 1991). Therefore, in these symptomatic individuals in whom no other bowel pathogen was detected, direct infection of the bowel mucosa by HIV-1 should be considered as pathogenic (Kotler *et al.*, 1991). Accordingly, the extensive diarrhoea and malabsorption observed in HIV-1-positive individuals could be the effect of toxicity of virus itself or viral proteins that disturb the intestinal cell membrane integrity, perhaps in the handling of sodium ions and water (Levy, 1998).

Most researchers believe that AIDS associated gastrointestinal symptoms might be an indirect effect of HIV-1 infection. There exist M-cells in the epithelium of intestine. These special intestinal epithelial cells function to bind intraluminal antigens, pass them to the macrophages of MIS and finally transport them to the enteric lymphoid tissue (DuPont & Marshall, 1995). Since HIV-1 has been detected in mononuclear leukocytes from the intestinal lamina propria of AIDS patients (Barnett *et al.*, 1991), it was hypothesized that HIV-1 may enter the MIS via M cells

to infect macrophages in the lamina propria and transinfect the lymphocytes with subsequent production of virus-specific IgA, the principal protective intestinal immunoglobulin (DuPont & Marshall, 1995). These infected macrophages and T cells could induce the production of cytokines that might be toxic for the bowel mucosa (Levy, 1998).

With the progression of infection, an increased number of CTLs has been found in the small intestine of HIV-infected patients (Snijders *et al.*, 1996). However the number of CD4+ T cells and antigen-presenting cells (APC) in the bowel decline with advancing infection (DuPont & Marshall, 1995; Veazey *et al.*, 1998). Whether the depletion of these cells is a cytotoxic effect of CTLs or other factors is not conclusively decided yet. The dysfunction and destruction of APCs (e.g., activated DC) and CD4+ T cells is believed to contribute to the dysfunction of MIS (DuPont & Marshall, 1995). As the disease advances, the number of CD8+ T cells also declines as they do in the peripheral blood, and the MIS finally collapses. Following systemic dysfunction of the immune system, the individual becomes more susceptible to intracellular pathogens (DuPont & Marshall, 1995).

1.7 Prevention and Therapeutics

The ways to prevent further HIV spread, and to treat those who have been infected, and thus to keep the HIV pandemic under control, include principally the modification of human behaviour, the discovery and development of antiviral therapies, and particularly the development of effective vaccines.

1.7.1 Modification of Human Behaviour

To alter human behaviour through education is a basic and effective method for preventing further spread of the HIV epidemic. Programmes for preventing the spread of HIV, led by the World Health Organisation (WHO), are divided into mass-awareness campaigns for the general public and interventions combined with supportive services targeted at specific groups (d'Cruz-Grote, 1996). Knowledge of

HIV and how to avoid it are the most important items of information in which people need to be educated. Also, correct and safe behaviours, such as a reduction in the number of sexual partners, and the use of condoms, need to be established to protect both at risk individuals and their partners. For high risk groups, such as homosexual or bisexual men and drug injectors, some successes have been achieved through providing cheap condoms, clean injection equipment, HIV testing and treatment; creating the environment where safer sexual or drug-taking behaviour can be discussed and acted upon; and arranging medical, social and mental health services for HIV-infected people (Coates *et al.,* 1996b; UNAIDS/WHO, 1998).

Although there is little firm evidence of impact on behavioural change, and the strategies are not efficiently practiced, it is clear that prevention does work (d'Cruz-Grote, 1996; UNAIDS/WHO, 1998). In some developing countries, behavioural surveys have shown that the use of condoms is becoming prevalent, and that more people are aware of the importance of having safer sex (UNAIDS/WHO, 1998). A decrease in HIV prevalence is predicted as a result, but will take time.

1.7.2 Antiretroviral Treatment for HIV Infection

For those people who are now living with HIV/AIDS worldwide, altering their risk behaviour or developing a vaccine is not a practical concept for them, and the only way forward is the discovery and development of effective treatment to delay the onset of disease or to prolong patients' survival time. The research efforts of scientists leading to soundly based knowledge of the molecular mechanisms in the HIV life cycle have provided an essential and useful basis for the design of antiviral drugs. Several steps in the replication of HIV have received close attention in this context (Table 1. 6). For the present, only the inhibitors of reverse transcriptase and of viral protein processing have been successfully applied in clinical therapy (Lipsky, 1996).

Table 1. 6. Opportunities for antiviral action. (From: Lipsky, 1996)

1.	Attachment of virus to the cell
2.	Inhibition of reverse transcriptase, which creates DNA from viral RNA
3.	Inhibition of RNase H, which degrades viral RNA after viral DNA has been synthesised
4.	Inhibition of viral integrase, which is used to integrate viral DNA into the cell's DNA
5.	Inhibition of expression of the HIV gene once it is integrated into the host-cell DNA, including the processes of transcription of more viral RNA and the translation of viral proteins
6.	Inhibition of processing and post-translational modification of protein products of the virus

1.7.2.1 Inhibition of Reverse Transcriptase

The first drug to be used in HIV infection was zidovudine (AZT), an inhibitor of RT. It is a nucleoside analogue and works as a DNA chain terminator. The replication of virus can be prevented since DNA synthesis is terminated, therefore causing viral stasis (Levy, 1998).

Initially, AZT was shown to improve survival in patients with AIDS and AIDS-related illnesses when large doses were taken (1500mg/day) without a break for up to 6 months, but this regime was usually associated with severe side effects, especially toxicity to the bone marrow (reviewed in Levy, 1998). Later, the Concorde trial, a three year clinical trial which involved several cohorts in Europe, showed that AZT was not effective in delaying the time of onset for AIDS or in increasing survival significantly (Lipsky, 1996). However, AZT is good in reducing maternal-infant transmission (Lipsky, 1996), and it shows great value in clinical combination with other drugs (reviewed in Montaner *et al.*, 1998a).

With time, four more nucleoside analogues, didanosine (ddI), zalcitabine (ddC), stavudine (d4T) and lamivudine (3TC), and three non-nucleoside RT inhibitors, nevirapine, delavirdine, efavirenz, were introduced into clinical treatment for AIDS and HIV infection. These drugs appear to be more effective and less toxic to bone marrow than AZT. However, they soon lead to the emergence of resistant viral strains and are also associated with unpleasant side effects such as neuropathy and pancreatitis (reviewed in Levy, 1998).

1.7.2.2 Inhibition of HIV Protease

HIV protease functions to cleave viral precursor proteins to yield mature protein products. This procedure is important for viral replication and any failure will lead to the production of non-infectious virions (Refer to section 1.2.3.2). Thus, the inhibition of HIV protease provides another therapeutic category for AIDS and HIV infection. At present, there are four protease inhibitors used clinically, including saquinavir, ritonavir, indinavir, and nelfinavir.

When used alone or in combination with RT inhibitors, these drugs markedly reduce the viral load and increased the number of CD4 cells, and also show improvement in survival (reviewed in Deeks *et al.*, 1997). Nevertheless, protease inhibitors are found to cause clinical side effects. Most often, HIV patients who received protease inhibitors develop hyperglycemia and diabetes. Also, these drugs are able to interact with various analgesics, antibiotics, anticoagulants and other antiretroviral drugs. Drug-resistant strains to these protease inhibitors usually emerge very quickly, and the use of protease inhibitors requires to be carefully regulated in an exacting therapeutic regime (Deeks *et al.*, 1997; Levy, 1998 and in).

1.7.2.3 Highly Active Antiretrovial Therapy

Because of the limitations of individual drugs, a new therapeutic strategy, highly active antiretroviral therapy (HAART) commenced in the summer of 1996. This new strategy utilised two or three drugs in combination, and aimed to decrease

the plasma viral load below the lower limit of detection.

Initially, two-drug therapy was used. Usually, two RT inhibitors were used together to increase the efficiency of the treatment. This dual-nucleoside therapy was abandoned because resistant strains quickly emerged and the persistent control of virus replication was not achieved (reviewed in Levy, 1998).

The more recent regimen is triple-drug therapy, usually involving two nucleoside analogues plus a protease inhibitor, or plus a non-nucleoside RT inhibitor. This strategy has been confirmed as effective in decreasing AIDS-related morbidity and mortality, and in achieving sustained and progressive immunological reconstitution in both early and late HIV-1 infection into the second year of HAART (Li *et al.*, 1998; reviewed in Montaner *et al.*, 1998a;). Also, the risk of developing resistance is reduced, because of the marked reduction of viral evolution during treatment with the current therapeutic strategy.

Although triple-drug therapy dramatically decreased the plasma viral load and effectively suppressed viral replication, the plasma concentration of HIV-1 proviral DNA was found to decrease only slowly. This could provide a basis for continued virus replication, and the rapid rebound of viral replication occurring after cessation of drug therapy (Montaner *et al.*, 1998b).

The safety of HAART is another cause for concern. More and more articles report the potential long-term adverse effects of protease inhibitors, including hypertriglyceridaemia, hypercholesterolaemia, hyperglycaemia, lipodystrophy (Carr *et al.*, 1998) and vascular complications (Henry *et al.*, 1998). In addition, HAART is expensive, and its cost is far out of reach for most HIV-infected people, especially for people living in less developed countries, which can scarcely afford to provide AZT. The fight against HIV still presents many challenges.

1.7.3 HIV Vaccines

Apart from altering human behaviour, the development of HIV-1 vaccines is thought to be the main hope of preventing further HIV-1 infection.

At the beginning of the HIV epidemic, much effort was devoted to searching for an effective vaccine. However, little success was achieved because of poor understanding of the interaction between protective immunity and HIV infection, and of the amazing mutational ability of HIV (Haynes, 1996).

Two recent studies show that neutralizing antibody against the HIV-1 envelope glycoprotein can completely block HIV-1/SIV chimeric virus infections of macaque monkeys (Igarashi *et al.,* 1999; Shibata *et al.,* 1999). In these two studies, macaques were passively immunised with purified immunoglobulin (IgG) from chimpanzees infected with several different HIV-1 isolates, then challenged intravenously with a chimeric simian-human immunodeficiency virus (SHIV) bearing an envelope glycoprotein derived from HIV-1_{DH12}. Complete protection was achieved, but only if a sufficient concentration of antibodies was infused. The ability of neutralising antibodies to prevent HIV-1 infection is becoming clear. Therefore, to induce an effective antibody response could be an important goal of those developing HIV-1 vaccines (Moore & Burton, 1999).

Although a wide variety of different vaccine strategies have been evaluated in the animal model of macaques, including the use of recombinant poxviruses, DNA vaccines, and whole inactivated virus, successes have not been claimed. Live attenuated SIV vaccines have proven to be the most effective approach to induce protection against challenge with pathogenic SIV, but the safety of this approach is in question (Johnson, 1999). Baba and colleagues started their live attenuated triple-deleted SIV vaccine studies in neonatal and adult macaques in 1995, and it proved to offer good protection at the beginning (Babe *et al.,* 1995). Unfortunately, their macaques developed AIDS three years after immunization with defective virus (Baba *et al.,* 1999).

The ideal vaccine should be the one that lacks toxicity, induces neutralizing antibodies, and provides long-lasting immune responses at mucosal sites as well as in the blood. Thus far, HIV-1 vaccine development is still in its early stages.

1.8 HIV-1 Infection in Edinburgh

The individuals observed in this study were from a cohort of predominantly drug using HIV-1-infected patients identified in Edinburgh. Epidemiologic studies have shown that these individuals were infected at approximately the same time (Robertson *et al.*, 1986), and detailed clinical follow up studies have been possible due to the relative coherence of this group of individuals and despite their social isolation (Bell *et al.*, 1996a; Brettle *et al.*, 1996; Goodwin *et al.*, 1996). Serologic analysis of stored serum samples indicated that HIV-1 was introduced into Edinburgh and spread rapidly in late 1983 and early 1984 (Peutherer *et al.*, 1985). This rapid spread of the virus in Edinburgh may be explained by the high-risk behaviour of the intravenous drug users (IDU) in Edinburgh, since at the time of this rapid spread there was a very high frequency of needle sharing. In addition, an extremely high level of HIV-1 encephalitis has previously been noted in IDU in the Edinburgh cohort once they progressed to AIDS (Bell *et al.*, 1996a).

The Edinburgh cohort provides an excellent opportunity to undertake systematic investigations of the pathogenesis of HIV-1 infection, due to the comprehensive clinical database and the availability of multiple tissue samples (Bell *et al.*, 1996b). Previously, studies have focused on the nervous system. In this thesis, I investigated various organs from individuals in different stages of disease, and from different risk groups. Both histological and virological examinations were undertaken in order to understand the evolution of HIV-1 infection.

1.9 Aims

For a better understanding of HIV-1 pathogenesis and the mechanisms underlying HIV-1 related disease in specific tissues such as the brain, lung and gastrointestinal system, this thesis was aimed to investigate the cellular localisation of HIV-1, cytopathology and viral phenotype variation in various organs throughout the course of HIV-1 infection. The studies included in this these are as follows:

A. To determine the cellular localisation of HIV-1 in various organs obtained from HIV-infected individuals at different stages of disease. Immunohistochemistry was employed for detection of HIV-1 viral proteins and identification of cellular targets, and histology for visualisation of pathological abnormalities.

B. To investigate the genetic relationships of HIV-1 variants derived from various organs within HIV-infected individuals. Nested PCR detecting HIV-1 proviral DNA was employed for the investigation of the distribution of HIV-1. Nucleotide sequencing followed by phylogenetic analysis was employed for the investigation of HIV-1 quasispecies derived from various organs at different stages of HIV-1 infection.

Chapter 2: General Materials and Methods

2.1 Study Material

All autopsy samples used in this thesis were obtained from the Medical Research Council Brain and Tissue Bank of Edinburgh (Western General Hospital, Edinburgh). Tissues from various organs were obtained from fifty autopsies from the cohort of HIV-1 positive individuals in Edinburgh. Twelve of the fifty patients died of non-HIV related causes before developing AIDS. No HIV related changes were observed on pathological examination of these cases, which were classified in the pre-symptomatic stage (pre-AIDS). The other thirty-eight individuals died of complications associated with HIV-infection, including opportunistic infections or neoplasms. These patients were classified in the symptomatic stage (AIDS). Both fresh frosen (-70^{o}C) and formalin-fixed, paraffin-embedded tissues were available in all cases.

Tissues from all of the study subjects were examined by immunohistological staining with anti-p24 antibody and several cell markers. In addition, HIV-1 sequence analysis was performed on nine autopsies, including six symptomatic and three presymptomatic cases. Clinical information for each study subject is summarised in the relevant chapters.

2.2 Autopsies

Autopsies were carried out within three days of death. At autopsy, approximately 1-2 cm^3 samples of various organs, including brain, lymph node, intestine, lung, spleen, liver and kidney from each individual were removed and stored at -70^{o}C for polymerase chain reaction (PCR), sequence analysis and cryostat studies. The remaining tissues were fixed in 10% formalin for later histological examination. Approximately two weeks after fixation, tissues were processed through a 41 hour program in the Vacuum Infiltration Processor (VIP) (Tissue Tek), followed by paraffin wax embedding using a Tissue Tek embedding console. Fixed

and processed tissues were thus rendered safe for study outside the Containment Level 3 Lab.

2.3 Biological Risks of Working with HIV-1

Human immunodeficiency virus type 1 (HIV-1) has been classified as a dangerous pathogen of the hazard group 3. All work with live HIV-1 must be carried out within a specifically designed Containment Laboratory. It is recommended that the growth and manipulation of HIV in research laboratories must be carried out in Containment Level 3 facilities.

HIV-1 is a blood-borne virus. Transmission of HIV infection may occur via contact with any body fluids of an infected individual which might contain virus particles or infected cells, such as cerebrospinal fluid, peritoneal, pleural, pericardial fluids, semen, vaginal secretions, breast milk and blood. Unfixed tissues, organs and any other body parts from HIV positive patients are also included in this high-risk categorization.

2.3.1 Safe Handling of HIV-1 Infected Tissues

Needle stick or scalpel blade injuries with contaminated items are the major laboratory infection route in the laboratory. Any cut with a contaminated object can result in the introduction of HIV into the blood or lymphatic systems with the concomitant risk of infection, thus special care must be taken while dealing with syringes, needles, scalpels and scissors. Appropriate safety measures should be employed. These include wearing double gloves, a plastic apron over a dedicated coat, and disposable plastic cuffs being worn at all times. Chain mail gloves between two layers of rubber gloves should be worn when undertaking cutting of fresh tissues. A face shield is also required to protect the eyes and mouth from splashes. All work involving blood, body fluid, or tissues from HIV-1 positive individuals, must be undertaken in a Containment Level 3 laboratory using a Class 1 microbiological

safety cabinet. Unfixed high-risk specimens must not be exposed outside the Class 1 safety cabinet. All work surfaces must be disinfected regularly and all sources of potential contamination eliminated as far as possible to ensure a safe working environment for all staff.

2.3.2 Decontamination of HIV-1 Infected Tissues

HIV must be inactivated before any sample is removed from the Containment Laboratory. There are several conventional methods that can be used to achieve this, such as sterilisation by moist heat, dry heat or by chemicals.

2.3.2.1 Chemical Disinfection

Chemicals can be used to destroy HIV-1. Up to 10^7 fold decreases in infectious ability can be achieved by the treatments listing in Table 2. 1 (Resnick *et al.*, 1986).

Table 2. 1. Chemical disinfections.

Chemical	Exposure Duration
0.5% (v/v) Nonidet P-40	1 minute
0.5% (w/v) Sodium hypochlorite	1 minute
70% (v/v) Ethanol	1 minute
0.08% (w/v) Quaternary Ammonium chlorides	10 minutes
1:1 (v/v) ethanol / acetone	20 minutes

In general, chemical treatments provide a quick and convenient method of disinfection, especially for the working surface or small equipment. However, the major disadvantage of chemical disinfection is the incomplete penetration throughout the contaminated sample. Chemical disinfection cannot therefore be relied upon to work adequately with precipitates or blood clots in the sample. For this reason, heat inactivation, such as autoclaving is always the method of choice for rendering materials stage for disposal, ideally followed by incineration of the autoclaved waste.

2.3.2.2 Autoclaving

HIV is very labile at high temperatures under wet conditions. Routinely used conditions for the sterilisation of HIV-1 infected material is listed in Table 2. 2. Autoclaving is used to decontaminate experimental waste, such as gloves, gowns, tissue residues and small pieces of equipment. Fumigation is preferred for decontaminating working space and large pieces of equipment, such as microtome.

Table 2. 2. Autoclaving Decontamination

Temperature (℃)	Times (Min)
115	30
121	15
126	10
134	3

2.3.2.3 Fumigation

Fumigation with formaldehyde, under moist conditions, is an effective method of decontamination for rooms and for equipment, which is unsuitable for autoclaving. Routinely, fumigating gas is produced by one portion of potassium chromate mixed with one portion of formalin, which will then fill the whole space to be disinfected, if left overnight. This method is commonly used to decontaminate the microbiological safety cabinets after work. Since ingestion or inhalation of formaldehyde is extremely toxic, it is necessary to have a safe method for venting the formaldehyde after use. Also, it is important not only to use sufficient formaldehyde to achieve an effective concentration for inactivation of HIV but also not too great an excess as the formaldehyde will polymerize into paraformaldehyde on surfaces. This can result in the partial obstruction of HEPA (high efficiency particulate absorption) filters, and also a continuing slow release of formaldehyde back into the room due to depolymerisation from upon the walls, with consequent irritation of the eyes and mucosa of workers.

2.4 Preparation of Cryostat Samples

This work was carried out in the Containment Level 3 lab in the Department of Pathology, University of Edinburgh under the supervision of Ms C-A McKenzie, and Ms F. Brannan.

Protective clothing was worn as described in section 2.3.1 before entering the lab. Frozen tissues from various organs were trimmed to appropriate sizes and mounted on chucks with Tissue-Tek O.C.T. adhesive. This was carried out inside the Class 1 safety cabinet, and samples were then transferred to the Bright's cryostat freezing microtome (–20℃). 5μm specimen sections were taken from each block and transferred on to positively charged slides (Superfrost Plus glass slides, BDH Laboratories companies). After fixation and disinfection with acetone for at least 10 minutes at room temperature, the slides were stored at -20℃ for further investigation in the routine laboratory. The used gloves, gowns and experimental waste were decontaminated by autoclave. The used chucks and forceps were decontaminated by immersion in detergent (Trigene) overnight. The cabinet and microtome were left for fumigating overnight. All work surfaces were wiped with Disinfectant Wipes, containing 70% alcohol.

2.5 Preparation of Paraffin Samples

All HIV infected tissues that are adequately fixed in formalin may be considered decontaminated and therefore safe to handle in a routine laboratory. Formailin-fixed, paraffin-embedded tissue blocks were cut at 3-5μm using a Reichert-Jung Biocut 2030 Rotary Microtome. Sections were then floated on to a water bath at 45°C, and mounted on to appropriate glass slides, such as 0.1% w/v Poly-L-Lysine (Sigma) coated slides or Superfrost Plus glass slides (BDH Laboratories companies), and then incubated at 37°C for over 24 hours.

2.6 Immunohistochemistry

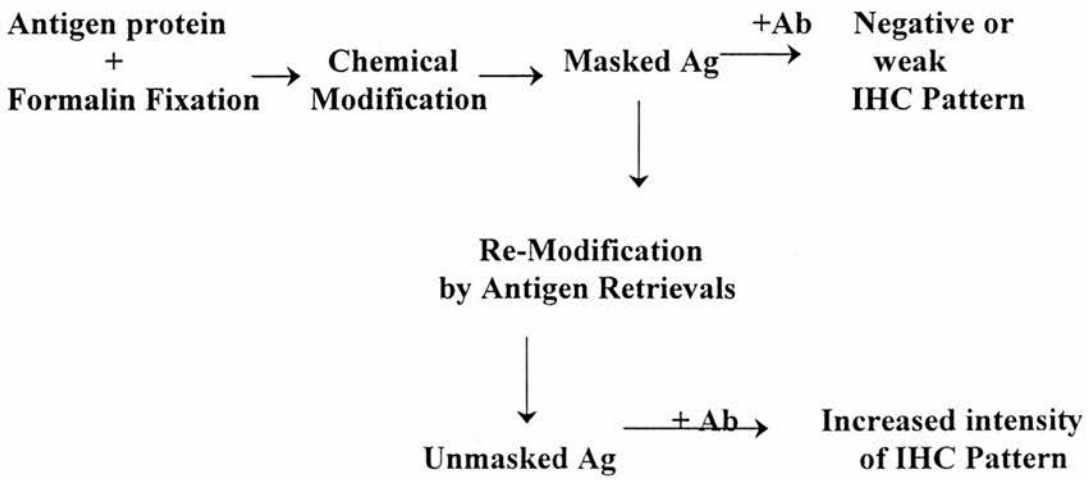
2.6.1 Rehydration of Paraffin-Embedded Sections

Sections were de-waxed in xylene (Genta Medical) for 10 minutes, and then rehydrated by passing through industrial methylated spirits (IMS) (Genta Medical) for 5 minutes, 70% alcohol (Hayman) for 5 minutes, and de-formalinised with saturated alcoholic picric acid buffer for 15 minutes. Finally, sections were placed in tap water until clear.

2.6.2 Antigen Retrievals

Formalin fixation provides the best morphological preservation, however it is believed that antigen identification in tissue sections is significantly limited by the masking effect of fixatives because of the formation of formalin-induced cross-linking proteins (Pileri *et al.*, 1997). This is thought to be the reason why some antibodies work well in paraffin sections, whilst others do not. One hypothesis, modification-re-modification raised by Shi *et al.*, suggested that the application of antigen retrievals may lead to a re-modification of the protein structure that had previously been modified when fixed with formalin, resulting in the reestablishment of the three-dimensional structure of antigenic epitopes in their active condition, or very close to that state (Shi *et al.*, 1997).

Several antigen retrieval techniques, including detergent treatments, proteolytic enzymes, and high temperature techniques were therefore developed since different antigens might only be exposed under particular conditions. The antigen retrievals described in the following sections were the ones most commonly used in this work. All of the conditions had been optimised and were applied routinely in the department of Neuropathology and Pathology in the Western General Hospital.



Key: Ag = Antigen; Ab = Antibody; IHC = Immunohistochemistry

Figure 2. 1. Diagram of Modification-re-Modification Hypothesis. During tissue fixation by formalin, the tertiary and quarter structure of cell surface markers or antigens might be changed because of so-called antigen masking effect. Such structural alternation will lead to an negative or weak IHC staining, however, using properly retrieval techniques, it is believed that the three-dimensional structure of these masked antigenic epitopes could be reestablished in their active condition, or very close to that state, allowing antibody detection, thus the intensity of staining could be increased. (Modified from Shi *et al.*, 1997).

2.6.2.1 Proteolytic Enzymes

Reagents:

- Trypsin (ICN Flow Laboratories)
- Calcium Choloride (Sigma)
- Protease (type XIV, 5 unit/mg, Sigma)
- 0.0125M TBS pH 7.8 --- made up by 1 volume of 0.125M Tris-HCL pH 7.6 and 9 volume of 0.85% (v/v) Sodium Chloride, using 1N HCl adjust the pH value.

Protocol:

Two solutions are available for antigen retrieval: **a.** 0.1% (v/v) Trypsin & 0.1% (v/v) Calcium Choloride solution, made up with 0.0125M TBS pH 7.8, or **b.** 0.5% (v/v) Protease solution, made up with 0.0125M TBS pH 7.8. The use of solutions is depended on the antibody (Table 2. 3).

Solutions for antigen retrieval were pre-warmed in a coplin jar, which was placed in a water bath at 37°C. De-waxed slides were proteolysed in this pre-warmed solution for 25-30 minutes at 37°C. The slides were then removed and rinsed in tap water for at least 5 minutes, in order to remove the emzymatic solution completely.

2.6.2.2 Microwave Irradiation

Reagents and Equipment:

- Citric Acid (Sigma)
- EDTA (Sigma)
- Microwave (600W)
- Proper container suitable for microwave

Protocol:

Two solutions are availed for Microwave irradiation: **a.** 0.01M Citric Acid buffer pH 6.0; **b.** 1mM EDTA buffer pH 8.0. The use of solution is depended on the

antibody (Table 2. 3).

De-waxed sections were placed in a microwave oven in a suitable container filled with either citric acid buffer or EDTA buffer, which depended on the primary antibody. The container was covered with cling film to prevent evaporation and microwaved for 15 minutes (3 X 5 minutes) at full power (600W). After microwave, the container was removed from the oven and left to cool at room temperature for 20 minutes. Sections were removed from the buffer and rinsed in tap water for at least 5 minutes to remove the microwave buffer thoroughly.

2.6.3 Blocking of Endogenous Biotin

Some tissues may non-specifically bind avidin, biotinylated horeseradish peroxidase or other Biotin/Avidin system components without prior addition of biotinylated antibody. This binding may be due to endogenous biotin or biotin-binding proteins, lectins, or non-specific binding substances present in the section. Vector Biotin blocking kit was required to block endogenous biotin, and these biotin-binding proteins and lectins, and thus reduce background staining.

Reagent:

- Biotin blocking kit (SP-2001, Vector Laboratories), which consists of an Avidin D solution and a Biotin solution.

Protocol:

After incubating with normal serum (SAPU), sections were then incubated with the Avidin D blocking solution for 30 minutes at room temperature. Rinsed briefly with washing buffer and then incubated with the Biotin blocking solution at room temperature for another 30 minutes. Rinsed thoroughly again with washing buffer prior to the addition of primary antibodies.

2.6.4 Immunostaining Techniques

The staining techniques used in this study were ordinary Avidin-Biotin Complex (ABC), immunofluorescence (IF), tyramide signal enhancement (TSA) and double-immunolabelling techniques. Staining results were then observed using a microscope. The imagines present in this thesis were taken using Leica Q5001W Analysis System (Leica Microscopy Systems, GmbH), and edited using Adobe Photoshop computer programming.

Reagents:

- Antibodies (summarised in Table 2. 3)
- TNB buffer --- 1M Tris-HCl pH7.5, 0.15M NaCl, 0.5% bovin-serum albumin (BSA)
- TNT Buffer --- 1M Tris-HCl pH7.5, 0.15M NaCl, 0.5% Tween®20
- Biotinyl-Tyramide reagent --- 10mg biotinamidocaproic acid 3-sulfo-*n*-hydroxysuccinimide ester (Sigma) and 3mg tyramine hydrochloride (Sigma), make up to 4ml with 50mM Borate buffer pH 8.0. Allow to conjugate with low-speed stir overnight, and filter for use.
- 50mM Borate buffer pH 8.0 & pH 8.5 --- 0.31g Boric Acid, make up to 100ml with distilled water. Adjust pH as required with NaOH

Protocol for Avidin-Biotin Complex (ABC) technique:

After rehydration, antigen retrievals were applied as required depending on the antibody we used (Table 2. 3). Sections were immersed in 3% H_2O_2 (Fisher Scientific International Company) for 10 minutes in order to block endogenous enzyme, followed by washing in tap water for 5 minutes. Sections were then blocked with normal serum (SAPU) for 10 minutes. Endogenous biotin was blocked using Vector Blocking kit (SP-2001, Vector Laboratories) as described in section 2.6.3, followed by primary antibody incubation. Details of the working conditions of antibodies are

summarised in Table 2. 3. After excess primary antibody was removed with washing buffer, sections then incubated with biotin labelled secondary antibody for 30 minutes at room temperature. Subsequently, the biotin site on the secondary antibody was bound by incubation with an enzyme labelled streptavidin-biotin complex (such as horseradish peroxidase (HRP)- or alkaline phosphatase (AP)-ABC, Dako) for another 30 minutes. The end point detection was by a colour reaction with a chromogen, such as diaminobenzidine (DAB), Vector Blue and Vector Red (Vector Laboratories), which gave dark brown, blue, red and purple colours respectively. After visulisation by chromogen, sections were counter stained in Meyer's haematoxylin for approximately 20 seconds and finally mounted with mounting medium (DPX). The results were observed by light microscope.

Protocol for Conventional Immunofluorescence (IF):

Same steps as ABC technique until finishing primary antibody incubation, but biotin blocking was dispensable. After primary antibody incubation, a fluorochrome (such as FITC) conjugated secondary antibody, was used instead of the biotin labelled secondary antibody for 30 minutes room temperature incubation. Sections were washed thoroughly with washing buffer and then mounted with vectashield fluorescence mounting medium (Vector Laboratories). These results were observed under an UV fluorescence microscope (fluoroscope).

Protocol for Tyramide Signal Amplification Technique (TSA):

Alternatively, after the incubation of biotin labelled secondary antibody, sections were blocked with TNB buffer for 30 minutes at room temperature, followed by incubation with HRP labelled streptavidin (SA-HRP, Dako) for 30 minutes. Excess reagent was washed away using TNT buffer, followed by incubation with biotinyl-tyramine reagent for 5 to 7 minutes. Washed thoroughly using TNT buffer, followed by 30 minutes incubation with enzyme labelled streptavidin (SA-HRP or SA-AP, Dako) at room temperature. End point detection was the same as ABC

method, using a chromogen (DAB, Vector Blue or Vector Red), followed by counter stained in Meyer's haematoxylin for approximately 20 seconds, mounted with mounting medium (DPX), and then observed by light microscope.

Protocol for TSA Immunofluorescence Technique (TSA-IF):

Same steps as TSA technique until finishing biotinyl tyramide incubation, followed by a fluorochrome conjugated streptavidin (SA-FITC, SA-Taxes Red). After 30 minutes incubation, sections were washed thoroughly with TNT buffer and mounted with vectashield fluorescence mounting medium. Then, observed under an UV fluorescence microscope.

Protocol for Double-immunolabelling Technique:

In general, the first antibody staining was carried out following the normal staining procedure until end point detection. After washed in buffer for at least 30 minutes, second antibody labelling was started from normal serum blocking, followed by normal staining procedure.

Table 2. 3. Information of antibodies

(A) Secondary Antibodies

Antibody	Source	Dilution
Biotinylated rabbit-anti-mouse (RAMBO)	Dako	1:200
Biotinylated swine-anti- rabbit (SARBO)	Dako	1:200
HorseRadish peroxdase labelled streptavidin	Dako	1:500
FITC-conjuated anti-mouse	SAPU	1:10
FITC-conjuated anti-rabbit	SAPU	1:10
Texas-Red conjugated streptavidin	Dako	1:500
FITC-conjugated streptavidin	Dako	1:500

(B) Primary Antibodies

Antibody	Source	Type*	Antigen retrieval	Method/ Dilution
CXCR4	Serotec	Mono	Protease/ 37°C, 30min	ABC --- 1: 100 TSA --- 1: 500
CCR-3	AIDS reagent project	Mono	Protease/ 37°C, 30min	ABC --- 1: 100 TSA --- 1: 500
CCR-5	R&D Systems	Mono	Protease/ 37°C, 30min	ABC --- 1: 50 TSA --- 1: 500
HIV-1 p24	DuPont	Mono	Microwave/ EDTA	ABC --- 1: 200 TSA --- 1: 3000
HIV-1 gp 41	Genetic System	Mono	Microwave/ EDTA	ABC --- 1: 100 TSA --- 1: 2000
CD3	Dako	Poly	Microwave/ EDTA	ABC --- 1: 150 TSA --- 1:1200
CD8	Dako	Mono	Microwave/ EDTA	ABC --- 1: 50 TSA --- 1: 800
CD21	Dako	Mono	Protease/ 37°C, 30min	ABC --- 1: 200 TSA --- 1: 1600
PG-M1(CD68)	Dako	Mono	Protease/ 37°C, 30min	ABC --- 1: 200 TSA --- 1: 6400
L26 (CD20)	Dako	Mono	N/A	ABC --- 1: 500 TSA --- 1: 16000
MB1	Euro-Path	Mono	Microwave/Citric acid	ABC --- 1: 50 TSA --- 1: 800
MT1 (CD45RO)	Euro-Path	Mono	Microwave/Citric acid	ABC --- 1: 150 TSA --- 1: 2400
UCHL1 (CD45RA)	Dako	Mono	Microwave/ Citric acid	ABC --- 1: 350 TSA --- 1:5600
GFAP	Dako	Poly	N/A	ABC --- 1: 2000 TSA --- 1: 32000

*Mono --- monoclonal antibody, which usually raised from mouse

Poly --- polyclonal antibody, which usually raised from rabbit.

2.6.4.1 CD4 Antibody

The CD4 molecule is confirmed as a major target of HIV-1 infection, and therefore of particular interested in this study. Unfortunately, antibody currently available did not provide reliable in paraffin sections in our lab despite rapid fixation. In order to investigate the association between p24 and CD4 positive cells, a short study using cryostat sections taken from known HIV-1 positive LNs have been made. In this case the DuPont p24 antibody was not detectable in our cryostat sections, for

reasons which are unclear but this was a consisted finding, and very few CD4 positive cells were detected in these sections.

Also, several p24 positive formalin-fixed, paraffin-embedded LN blocks have been sent to other research group who has reported that CD4 antibody working well in paraffin section. However, they were unable to have any CD4 staining in those sections, either. Therefore, the detection of CD4+ cells was unavailable in the present study.

2.7 Nucleotide Analysis

2.7.1 Extraction of Nucleic Acids

Reagents:

- TNE buffer --- 0.11M NaCl, 55mM Tris pH 8.0, 1.1mM EDTA pH 8.0, 0.55% sodium-*n*-lauroylsarcosine (SDS)
- Lysis buffer --- 0.11M NaCl, 50mM Tris pH 8.0, 1.1mM EDTA pH 8.0, 0.5% SDS, 100μg/ml Proteinase K, 40μg/ml poly A

Protocol:

0.5-1 cm^3 blocks of tissue were dissected from frozen material and placed into a clean eppendorf tube with 500μl lysis buffer, mixed well by vortexing, and then incubated at 65°C for two hours or until the tissue was completely dissolved. Following this, 450μl phenol (Rathburn Chemicals Ltd) was added to the extraction eppendorf and the contents mixed thoroughly by vortexing for 5 minutes. The extraction eppendorf was centrifuged at 13000rpm at room temperature for 10 minutes. The upper aqueous layer was transferred carefully without disturbing the interface into a clean eppendorf and phenol extraction was repeated as above. The upper aqueous layer was again transferred carefully to a clean eppendorf with 450μl of 50:1 (v/v) chloroform / iso-amylalcohol (AnalaR), mixed thoroughly by vortex for

2 minutes and then centrifuged at 13000 rpm at room temperature for 10 minutes. The aqueous layer was then transferred again to a clean eppendorf containing 800μl of 100% ethanol (AnalaR) and 40μl 3M NaOAc pH 5.2, mixed well by inverting and then left overnight at -20°C for precipitation of nucleic acid. Nucleic acid was collected by centrifugation at 13000rpm at 0°C for 30 minutes. The supernatant was discarded and the pellet was washed with 70% alcohol then centrifuged again at 0°C for 10 minutes. The supernatant was discarded and the pellet was dried at 37°C for 5 to 10 minutes. The pellet was re-suspended in 40μl of DEPC water. The sample was left at room temperature at least for 10 minutes to ensure adequate solubilization of the pellet.

2.7.2 Quantitation of Extracted DNA

The concentration of DNA in each sample was quantified using spectrophotometric UV absorbance readings at wavelengths of 260nm and 280nm. Samples were diluted 1 in 200 with DEPC water and absorbance measured in a spectrophotometer. The ratio of optical density (OD) at 260nm to 280nm gave an indication of purity of each preparation. An A_{260} /A_{280} ratio of approximately 1.8 indicated a relatively pure DNA preparation. The concentration of DNA was calculated from the equation: $A_{260} \times D \times 50$ = DNA concentration (μg/ml), where A_{260} = OD value at 260nm, D is the dilution factor (=200) and 50 is equivalent to the concentration (μg/ml) of double-strand DNA at A_{260} of 1.0.

2.7.3 Polymerase Chain Reaction

HIV-1 proviral DNA was amplified employing the nested polymerase chain reaction (PCR). Nested PCR involved a double round of PCR with the primary PCR amplification by an outer set of specific primers, followed by an secondary PCR using an inner set of specific primers, which was used to amplify the first PCR product. Compared with using only one set of primers, nested PCR can increase the

sensitivity of amplification by 10,000 times, and can also increase the specificity.

Reagents:

- 10X PCR reaction buffer --- 200mM Tris-HCl solution pH 8.8, 500mM KCl, 15mM $MgCl_2$ and 0.5% Triton X-100 (Promega)

Protocol:

Primary PCR using the outer set of primers was carried out in a 50μl volume. Each reaction contained 1μg of DNA, 0.4 units of *Taq* polymerase (Promega), 5μl of 10X PCR reaction buffer, 30μM of nucleoside triphosphate (dGTP, dATP, dTTP, dCTP) (Boehringer Mannheim), and 0.25μM of each of the outer nested primers, finally made up to 50μl with DEPC water. The secondary PCR, using an inner set of primers, was carried out in a 20μl volume. 1μl of primary PCR product was transferred to a second tube containing 19μl of reaction mixture of which conditions were the same as for the primary reaction. One to two drops of mineral oil was used to cover each reaction prior to transfer to the thermal cycler, in order to prevent loss of sample due to evaporation.

Both primary and secondary PCR were subjected to a thermal cycle of denaturation at 94°C for 36 seconds, primer annealing at 50°C (for V3 region) or 55°C (for $P17^{gag}$ region) for 42 seconds, and an extension step at 72°C for 90 seconds. Each template strand was amplified for 30 cycles in the primary reaction, and 25 cycles for secondary reaction. At the end of the last cycle in every reaction, samples were heated at 72°C for 5 minutes for terminating uncompleted strands. The nucleotide sequences of PCR primers are described in Table 2. 1. All positions are numbered according to the HXB2 genome (Myers *et al.*, 1991).

Known positive and negative DNA samples (1μg) were included in every reaction to provide a comprehensive check for contamination. An additional negative control was included which contained no DNA in the reaction mixture ensuring no

buffering contamination occurred.

Table 2. 4. Primer sequences

Primer	Sequence 5' to 3'	Position of 5' base
$P17^{gag}$ (sense)	GCG AGA GCG TCA GTA TTA AGC GG	795
$P17^{gag}$ (sense)	GGG AAA AAA TTC GGT TAA GGC C	835
$P17^{gag}$ (antisense)	CTT CTA CTA CTT TTA CCC ATG C	1248
$P17^{gag}$ (antisense)	TCT GAT AAT GCT GAA AAC ATG GG	1296
V3 (sense)	TAC AAT GTA CAC ATG GAA TT	6957
V3 (sense)	TGG CAG TCT AGC AGA AGA AG	7009
V3 (antisense)	CTG GGT CCC CTC CTG AGG	7331
V3 (antisense)	ATT ACA GTA GAA AAA TTC CCC	7381

2.7.4 Visualisation of Amplified PCR Products

Reagents:

- 10X TBE --- 108g Tris base (AnalaR), 55g boric acid (Molecular Biology certified; Kodak), 40ml 0.5M EDTA (Molecular Biology certified; Kodak). Make up to 1 litre with distilled water
- Agarose gel --- agarose (Sigma) make up with 1X TBE containing ethidium bromide.

Protocol:

2 to 3 % agarose gel containing ethidium bromide was used to demonstrate PCR results. Ethidium bromide as a DNA intercalating agent, which exhibits fluorescence under ultraviolet light, allowing any positive PCR products to be detected as bright fluorescent bands. Agarose gel was run for 20 minutes at 150V and analyzed under ultraviolet light.

2.7.5 Quantification of HIV-1 Proviral DNA

Quantitation of proviral DNA was carried out using the nested PCR limiting dilution method (Simmonds *et al.*, 1990). The number of copies of provirus per million cells was determined by nested PCR amplification of serial ten-fold dilutions of DNA using the $p17^{gag}$ primers. Ten replicates at last positive dilution were used to indicate the minimum proviral load in the sample, assuming a Poisson distribution for each sample by $-\ln(1-p)/d$ (where p= proportion of positive samples and d= dilution). Viral load was expressed as copies per million cells on the basis that a human diploid cell contains 6.6pg DNA.

2.7.6 PCR Product Cloning

One of the benefits of the nested PCR is producing a clear and unique band as a positive result. This PCR product was available for direct cloning using a Promega pGEM-T Easy Vector system, followed by transformation using blue/white colony screening.

2.7.6.1 Ligation

Ligation reactions were set up based on Promega T4 DNA Ligase system and pGEM-T Easy Vector system (Promega). The reactions were set up as in Table 2. 5. Mix the reaction by pipetting and incubate the reaction overnight at 15 °C.

Table 2. 5. Ligation reaction

	Standard Reaction	Positive Control
T4 DNA Ligase 10X Buffer	1µl	1µl
PGEM-T Easy Vector (50ng)	1µl	1µl
PCR product	1µl	-
Control Insert DNA (4ng/µl)	-	2µl
T4 DNA Ligase (3 Weiss units/µl)	1µl	1µl
Deionised water to a final volume of	**10µl**	**10µl**

2.7.6.2 Transformation

Reagents:

- SOC Medium pH 7.0 (100ml) --- 2.0g Bacto®-tryptone, 0.5g Bacto®-yeast extract, 1ml 1M NaCl, 0.25ml 1M KCl, 1ml 2M Mg^{2+} Stock (1M $MgCl_2 \cdot 6H_2O$, 1M $MgSO_4 \bullet 7H_2O$), 1ml 2M Glucose
- LB medium pH 7.5(1L) --- 10g Bacto®-Tryptone, 5g Bacto®- yeast extract, 5g NaCl
- LB agar pH 7.5 (1L) --- 10g Bacto®-Tryptone, 5g Bacto®- yeast extract, 5g NaCl, 15g agar

Protocol:

JM109 Competent Cells (Promega) were employed for transformation. The blue/white colony method was used as a screening method for transformation. 2μl of each ligation reaction was added to sterile 1.5ml eppendorf on ice. A known quantity of purified, supercoiled plasmid DNA was set up as a transformation control. 50μl of competent cells were carefully aliquoted into each eppendorf, mixed gently and placed on ice for 20 minutes. The cells were heat shocked at exactly 42°C in a water bath for exactly 45 seconds. The eppendorf was then returned immediately to ice for 2 minutes. 950μl of SOC medium was added to the tubes containing cells that had already transformed with ligation reactions. The transformed cells were grown for 1 to 2 hours at 37°C. 100μl of each transformed culture mixture was plated on to LB/ampicillin/IPTG/X-Gal plates. The culture mixture was left to stand at room temperature for 10 to 15 minutes and then incubated overnight at 37°C. After overnight culturing, these pates were stored at 4°C ready for miniprep. Generally, white colonies contained inserts.

Ligation Positive Control

To determine whether the ligation was proceeding efficiently, a positive control was always included as a standard reaction. Theoretically, more than 60% of the

colonies should be white and ideally less than 30% of the colonies should be blue. Those blue colonies were a useful internal transformation control. If no colonies were obtained finally, the transformation had failed. If only blue colonies were obtained, without whites, this suggested that the ligation step had failed.

Transformation Control

To determine the transformation efficiency of the competent cells, an uncut plasmid (supercoiled plasmid DNA) was used as a control. cfu/μg-DNA was calculated in the transformation control plate. If a figure of lower than 1x 10^8 cfu/μg-DNA was obtained, the reaction was deemed inefficient and it was necessary to prepare fresh competent cells in order to repeat the entire process.

2.7.7 Plasmid DNA Minipreps

Reagents:

- GTE (100ml) --- 0.9g Glucose, 2ml of 0.5M EDTA, 2.5ml of 1M Tris-HCl pH8
- Solution I --- GTE with Lysozyme (Sigma)
- Solution II (10ml) --- 400ul of 5M NaOH, 1ml of 10% SDS
- Solution III (100ml) ---9.5ml glacial acetic acid adjust the pH to 4.8 with KOH
- TE --- 10mM Tris-HCl, pH 8.0, 1mM EDTA

Protocol:

3 to 4ml of LB medium containing 100μg/ml of ampicillin were inoculated with a single, white bacterial colony which was then cultured in a 15ml sterile tube to ensure adequate aeration. The bacterial culture was incubated at 37°C with rigid agitation for 12-16 hours.

After 12-16 hours, 1.5ml of bacterial culture medium was transferred into an eppendorf and centrifuged at 12000 xg for 5 minutes. The supernatant was discarded into the waste bottle containing 10% HCl, and then pellets resuspended in 100ul of

Solution I. This destroyed the bacteria exposing their DNA. 200 µl of freshly made Solution II was added to the eppendorf that contained well-mixed solution I, and the mixture inverted. The mixture was allowed to stand on ice for 5 minutes, then 150 µl of ice cold solution III was added to precipitate the bacterial protein debris. After mixing well the mixture was allowed to stand on ice for another 5 minutes to complete the precipitation then spun down at 13000 rpm for 10 minutes.

The top aqueous layer was carefully transferred to a tube, which contained 290µl isopropanol (AnalaR), gently mixed and incubated at room temperature for at least 30 minutes for DNA precipitation. The precipitated DNA was centrifuged at 13000 rpm for 20 minutes. The supernatant was discarded and the pellet was washed with 500ul of ice cold 70% ethanol. The DNA was then dried at 37 °C for 10 to15 minutes and finally resuspended in 40ul of TE, containing 20ug /ml RNAse and stored at -20°C.

2.7.8 Nucleotide Sequence

The sequence reaction used was the T7 Sequenase version 2.0 DNA polymerase system (Amersham, Life Science). This system was modified from the bacteriophage T7 DNA polymerase. Initially, a pair of primer was annealed into the DNA template, followed by radiolabelling the annealed primer. Finally, a very fast elongation was processed and every reaction terminated by the addition of stop solution.

2.7.8.1 Pre-sequence Denaturation

Reagents:

- Deneturation buffer --- 2M NaOH and 2µM of EDTA pH 8.0

Protocol:

2µl of denaturation buffer was mixed well with 18µl DNA. This mixture was incubated at 37°C for 30 minutes. The denatured DNA was then precipitated by

adding 0.1 volume of 3M NaOAc pH 5.2 and 3-4 volume of 100% ethanol, and incubated at -20°C for at least 3 hours. Precipitates were then collected by centrifugation at 13000rpm for 30 minutes at 4°C. The supernatant was carefully discarded, the pellet was washed with 70% ice cold ethanol and then centrifuged at 13000rpm at 4°C for another 20 minutes. After discarding the supernatant the pellet was dried at 37°C for 5 to 10 minutes and re-dissolved in 13μl distilled water. The sample was now ready for sequencing.

2.7.8.2 Annealing

After pre-sequence denaturation, DNA was ready for primer annealing. For each template, the annealing reaction was set up as Table 2. 6, and the nucleotide sequences of primers were listed in Table 2. 7. Each reaction was mixed well, pre-warmed at 65°C for 2 minutes, and then cooled slowly to room temperature. Briefly centrifuge these reaction tubes to collect the contents after they returned back to room temperature.

Table 2. 6. Annealing reaction

	Forward Reaction	**Reverse Reaction**
Primer	0.5 pmol	0.5 pmol
DNA	6.5μl	6.5μl
5X T7 sequenase reaction buffer	2μl	2μl
Adjust total volume to 10μl with dH2O	**10μl**	**10μl**

Table 2. 7. Primers for sequence

Direction	Sequence	Primer binding site on pGEM-T Easy Vector
Forward	GTTTTCCCAGTCACGAC	2959-2975
Reverse	GTCATAGCTGTTTCCTG	176-192

2.7.8.3 Labelling and Termination Reaction

Reagents:

- 5X sequenase reaction buffer --- 200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl
- 5X labelling mixture --- 7.5μM dGTP, 7.5μM dTTP, 7.5μM dCTP, 5μCi [α-^{35}S]dATP
- Termination nucleotide mixture --- 80μM dATP, 80μM dTTP, 80μM dGTP, 80μM dCTP
- Stop solution --- 95% formamide, 20mM EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue

Protocol:

The labelling reaction was set up as shown in Table 2. 8. Because T7 DNA polymerase is temperature sensitive, the procedure must be carried out rapidly after addition of the T7 DNA polymerase. 4 tubes were labelled G, A, T, C, and filled with 2μl of the appropriate dideoxy termination nucleotide and 0.5μl of DMSO. These tubes were pre-warmed at 37°C and 3.5μl of labelling mixture were transferred into each tube. After 2-4 minutes incubation at 37°C, 4μl of stop solution were added to terminate the reaction. These reactions were ready for loading on to the gel.

Table 2. 8. Labelling reaction

Annealing reaction mixture	10µl
0.1M DTT	1µl
1X Labelling Mix (diluted from 5X Labelling Mix with dH_2O)	2µl
[α-^{35}S] dATP	0.5µl (0.5µCi)
T7 Sequenase DNA polymerase	3.25 units
Total	**15.5µl**

2.7.8.4 Acrylamide Gel Electrophoresis

Reagents:

- 10X Sanger TBE --- 324g Tris base, 85g boric acid, 19g EDTA, make up to 2L with distilled water
- 6% Acrylamide gel --- 21g Urea (AnalaR), 6ml 50% Long Ranger gel solution (Flowgen), 5ml 10X Sanger TBE, 0.05g APS (Sigma), make up to 50ml with distilled water

Protocol:

The sequencing reactions were run on a 6% acrylamide gel. Glass plates were cleaned with Methanol then acetone. A pair of flat spacers was used to assemble the plates. The gel mixture was poured carefully to avoid producing bubbles and left to polymerise for at least 2 hours.

The gel was pre-run at 75 volts for 10 minutes using 1X Sanger TBE as the electrophoresis buffer. The samples were heated to 90-95^{o}C for 5-10 minutes then loaded on to the gel. The gel was run at 75 volts. After electrophoresis, the gel was disassembled and dried, followed by exposed to X-ray film for 1-2 days, depending on the density of signal.

2.7.9 Analysis of Sequence Gel

Sequencing gels were read and aligned using Simmonic 2000 Software, which was developed in the department by Dr. P. Simmonds. Sequence data were then analysed using the computing package MEGA (Molecular Evolutionary Genetics Analysis). MEGA is one of the computer programs for analyss data from DNA, RNA and protein sequences, and distance matrices. Also, it includes the neighbour-joining method, distance matrix method, a branch and bound parsimony method, and bootstrapping.

Phylogenetic trees were constructed using the HIV HXB2 sequences as the outgroup unit (OUT), and the Jukes-Cantor method was employed to count the multiple substitutions. The bootstrap resampling method (100 replicates) was used to assess the confidence of each node in the tree constructed.

Chapter 3: Development and Optimisation of The Tyramide Signal Amplification Method in The Detection of HIV-1 Viral Proteins

3.1 Introduction

3.1.1 HIV-1 Detection and Immunohistochemistry

Acquired immunodeficiency syndrome (AIDS) was first described in homosexual men and intravenous drug users during 1981 and 1982 (refer to section 1.1). Several epidemiologic studies then indicated that the pathogen of AIDS, later termed human immunodeficiency virus (HIV), was transmitted mainly by intimate sexual contact and contaminated blood (Jaffe *et al.,* 1983). Because of concern generated by the reported transmission of AIDS by contaminated blood, blood products and contaminated medical equipment, major efforts were made early in the study of HIV-1 to quantify virus in blood samples.

The detection techniques for HIV-1 in blood and other body fluids include the measurement of viral p24 antigen (Goudsmit *et al.,* 1987a), and of HIV-1 RNA by reverse-transcriptase polymerase chain reaction (RT-PCR) (van Kerckhoven *et al.,* 1994) and quantitative competitive PCR (QC-PCR) (Menzo *et al.,* 1992), and of the detection of HIV-1 proviral DNA by PCR. The results of these studies indicated that both free virus particles and infected cells were present in blood, and HIV-1 infected cells appeared to be more commonly found than infectious virus particles (Embretson *et al.,* 1993b). These virus-infected cells were observed to remain in the human body longer than free virus particles, and normally serve as a continual source of new virions (Levy, 1998). It was found, also, that transmission by these virus-infected cells to new cells was much more effective than by free virus particles (Levy, 1998). All this evidence suggested that HIV-1 infected cells might be more important in the transmission and progression of the disease than free virus particles. Therefore, it was postulated that investigation of the localisation and classification of these HIV-1 infected cells, especially their distribution in organs, might assist in the understanding of HIV-1 pathogenesis.

There have been a small number of studies devoted to the investigation and quantification of HIV-1 in a variety of organs, employing the PCR technique (Donaldson *et al.*, 1994a; Nakata *et al.*, 1995). This gene amplification technique has

been widely used as the most powerful tool in rapidly detecting virus or rearranged genes, and allowed the detection of low-level viral RNA and DNA. However, based on the presence of HIV-1 nucleic acid, it has proved difficult to correlate PCR results with the pathological features of the material being tested. In order to demonstrate productive infection and cellular localisation of HIV-1, the application of immunohistochemical staining for viral antigens was believed to be the best method.

Immunohistochemistry (IHC) is an indispensable technique in investigative and diagnostic histology and pathology. The reagents and protocols for different IHC applications are improving rapidly. Initially, the usage of IHC was limited by the sensitivity of the staining technique. Soon the production of monoclonal antibodies and the introduction of indirect staining techniques, from the three-stage immunoperoxidase or alkaline phosphatase staining technique to the five-layer immunostaining procedure (Peroxidase-Anti-Peroxidase (PAP), or Alkaline Phosphatase-Anti- Alkaline Phosphatase (APAAP)), greatly enhanced the staining signal. A more exciting development was the discovery of the biotin-avidin complex assay (ABC) (Hsu *et al.,* 1981). It was found that biotin can be attached to a variety of compounds and then simply detected by binding it with high affinity and specificity to avidin that was conjugated with an enzyme or a fluorochrome (Adams, 1992). This practical application of the biotin-avidin system not only gave a remarkable improvement of staining sensitivity, it also provided a useful basis for developing other enhancing techniques.

Following the improved staining technique of IHC, another problem, the so-called antigen-masking effect of fixation, remained to hinder the methodology. For histological examinations, formalin is the general fixative of choice. However during the process of formalin fixation, the tertiary and quaternary structures of tissue proteins are believed to be altered by extensive cross-linking, while the primary and secondary protein structures are well preserved by aldehyde-linked protein aggregates (Merz *et al.*, 1995). The cross-linking barriers are presumed to restrict the accessibility of antigenic epitopes. Many methods have been used in an attempt to retrieve the antigenicity from formalin-fixed tissues, including treatments with

detergents, trypsinization or the use of other proteolytic enzymes, and the use of formic acid (Munakata & Hendricks 1993; Merz *et al.*, 1995; Pileri *et al.*, 1997; Shi *et al.*, 1997). A more recent development has been the use of microwave irradiation (McQuaid *et al.*, 1995; Shi *et al.*, 1997). Its ability to contribute to maximum immunoreactivity in formalin-fixed tissues has greatly increased the range of histological investigations possible with IHC.

Bringing these improvements of staining and retrieval techniques together, IHC has now been widely applied in many investigative and diagnostic fields. However, in the field of HIV-1 detection, there has been a shortage of optimal procedures and antibodies, and the application of IHC in specific detection of HIV-1 viral proteins was not well developed, particularly for tissues containing a low viral load.

3.1.2 Anti-HIV-1 Antibodies

The design of antibodies for HIV-1 diagnosis first focused on the HIV-1 core p24 protein (Langedijk *et al.*, 1990). HIV-1 p24 protein is encoded by the *gag* gene. It is the major component of the viral capsid, and its amino acid sequence is highly conserved (refer to section 1.2.3.1). In clinical practice, the HIV-1 p24 antigen is one of the markers for measuring viral activity (Fahey *et al.*, 1990; Langedijk *et al.*, 1990) and a useful tool for monitoring antiviral drug therapy (Goudsmit *et al.*, 1987b; Andrieu *et al.*, 1988). A more important finding is that an elevated level of HIV-1 p24 antigen or infectious virus in the blood has been proved to link with HIV-related disease progression (Allain *et al.*, 1987; Tsiquaye *et al.*, 1988; Sei *et al.*, 1989; Fahey *et al.*, 1990; Ujhelyi *et al.*, 1990; Chargelegue *et al.*, 1995).

In the 1980s, the DuPont Company registered the first success in antibody development for HIV-1 immunoassay, an anti-p24 antibody for an enzyme-linked immunosorbent assay (ELISA) assay (DuPont HIV p24 Core Antigen ELISA kit). Subsequently, other anti-p24 antibodies, reacting against different epitopes or raised in different species, were designed and employed in serological antigen capture studies, such as ELISA, enzyme immunoassays (EIA) and Western Blot (Baroni *et al.*, 1986; Pumarola-Sune *et al.*, 1987; Housset *et al.*, 1990). However, an antibody

which was suitable and consistently effective for examining formalin-fixed tissues did not become available until the late 1980s when the DuPont company introduced an advanced HIV-1 monoclonal antibody (mAb) (NEA-9283) to p24 core protein for paraffin embedded tissues. This commercially available anti-HIV-1 p24 monoclonal antibody required a pepsin retrieval procedure to unmask antigen, and overnight incubation for the primary antibody. Although it did have better labeling ability in a comparison study with other anti-p24 mAbs (Cartun *et al.,* 1988), the method was time-consuming, and the presence of greater background staining due to overnight incubation limited its application. Later studies reported that this antibody did not work in paraffin-embedded brain tissues in their trials, suggesting that this antibody did not work consistently despite pepsin retrieval and overnight incubation (Kure *et al.,* 1990).

At the same time, Genetic Systems designed a mouse monoclonal anti-gp41 antibody for HIV-1 detection (Genetic Systems, Seattle, WA). It was raised against the relatively conserved portion of the HIV-1 envelope protein, gp41. This antibody also required an overnight incubation but no antigen retrieval. It gave apparently reproducible and consistent staining results. Therefore, this anti-gp41 mAb was frequently employed in the subsequent HIV-1 IHC studies at this stage possibly because difficulties had been encountered in p24 IHC (Chad *et al.,* 1990; Kure *et al.,* 1990). However, this monoclonal antibody showed some cross-reaction with an irrelevant epitope in Alzheimer neurofibrillary tangles (Kure *et al.,* 1990; Morgello *et al.,* 1998), and stained occasional macrophages in multiple sclerosis lesions (Sinclair & Scaravilli, 1992). The specificity of this anti-gp41 mAb is questionable.

Other immunohistochemical studies, most of them performed in animal models, which detected other HIV-1 viral proteins, such as Nef, Tat, and envelope gp120 (Ranki *et al.,* 1995; Lin *et al.,* 1997) were published occasionally. However, the staining results were not always reproducible.

Recently the development of antigen retrieval by microwave irradiation provided a dramatic improvement in immunohistochemical staining for HIV-1 viral

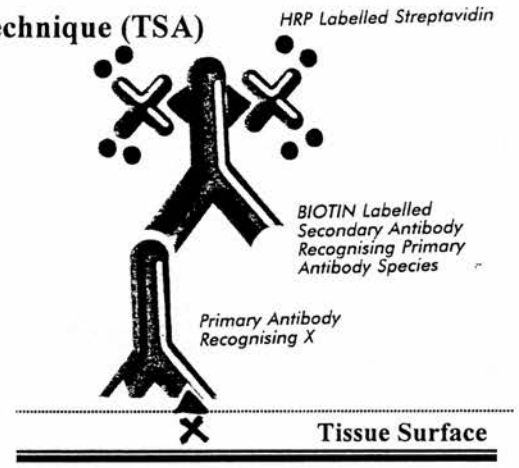
proteins. It helped to shorten the incubation period of the primary antibodies, for both anti-p24 (DuPont) and anti-gp41 (Genetic Systems) antibodies, and also increased the staining density. With the application of microwave irradiation in our lab, the DuPont anti-HIV-1-p24 mAb gave consistent and reproducible results with the routine ABC technique, although positive cells were rare in some tissues. Initial studies in our lab showed that, in correlating p24 IHC results with quantitative PCR within the same tissue, most of the PCR positive tissue blocks proved to be p24 IHC negative even when the PCR proviral loads were high (Donaldson *et al.*, 1994a). It was not clear whether these negative results were due to low or absent p24 antigen, or to the limitation of routine IHC staining technique (ABC method) in use at that time. In addition to the application of microwave retrieval, a signal-enhanced technique was required to investigate this problem.

3.1.3 Study Objectives

Based on a novel tyramide signal amplification system which was established by Bobrow *et al.* (Bobrow *et al.*, 1989; Bobrow *et al.*, 1991), this study was aimed to develop a more satisfactory and sensitive IHC technique for HIV-1 immunohistochemistry using formalin-fixed paraffin-embedded tissue samples.

Tyramine amplification technique is a further important development for IHC, which gives a promising improvement in sensitivity and extends the possible applications of IHC to new targets. In this signal enhancing technique, which was originally termed catalyzed reporter deposition (CARD), Horseradish-Peroxidase (HRP) was employed to catalyze the phenolic portion of biotinylated tyramide with the help of hydrogen peroxide (Bobrow *et al.*, 1992). This reaction produced highly reactive tyramide radicals that then bound covalently to electron-rich moieties (i.e. tyrosine and tryptophan) contained in tissue protein molecules. According to the very short half-life of tyramide radicals, the deposition occurred extremely close to the activating enzyme site. This property made it very useful in providing a localised staining pattern (Bobrow *et al.*, 1992). The biotin on the bound tyramide could then be visualised either by fluorochrome or by enzyme labelled streptavidin.

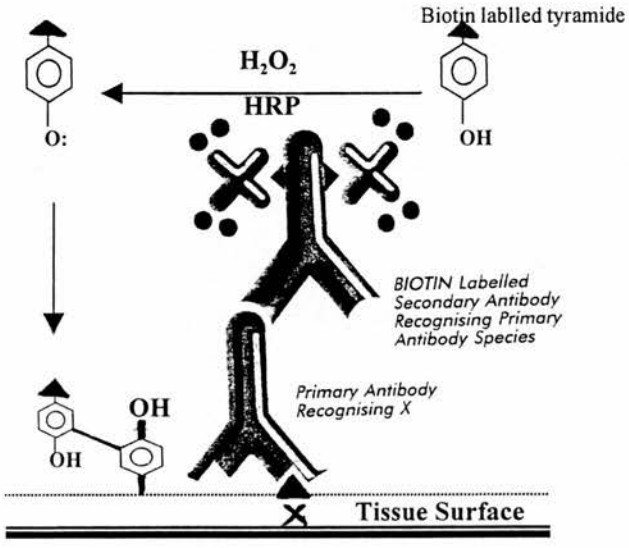
(a) Tyramide Signal Amplification technique (TSA)



Stage 1. General labelling procedure

1. Primary antibody recognising specific Antigen X
2. Biotinylated secondary antibody recognising primary antibody species.
3. HRP labelled streptavidin bound to biotin.

Stage 2. Increased biotin sites



4. Catalytic deposition of biotinyl-tyramide by HRP and H_2O_2
5. Covalent deposition of biotinyl-tyramide onto surfaces blocked with proteins close to the enzyme site.
6. Resulted in increased biotin sites

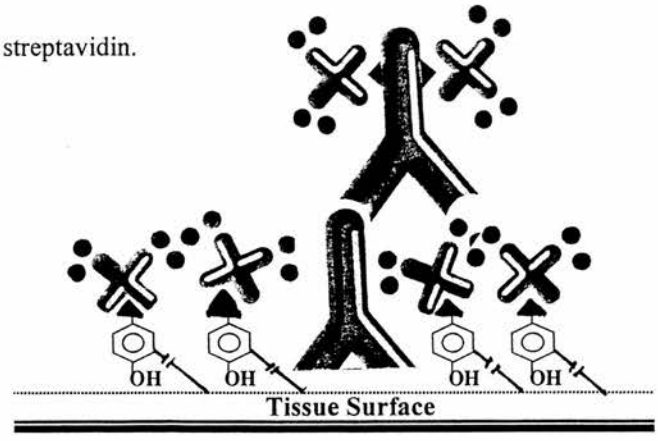
Stage 3. End point detection

***For colour reaction*:**

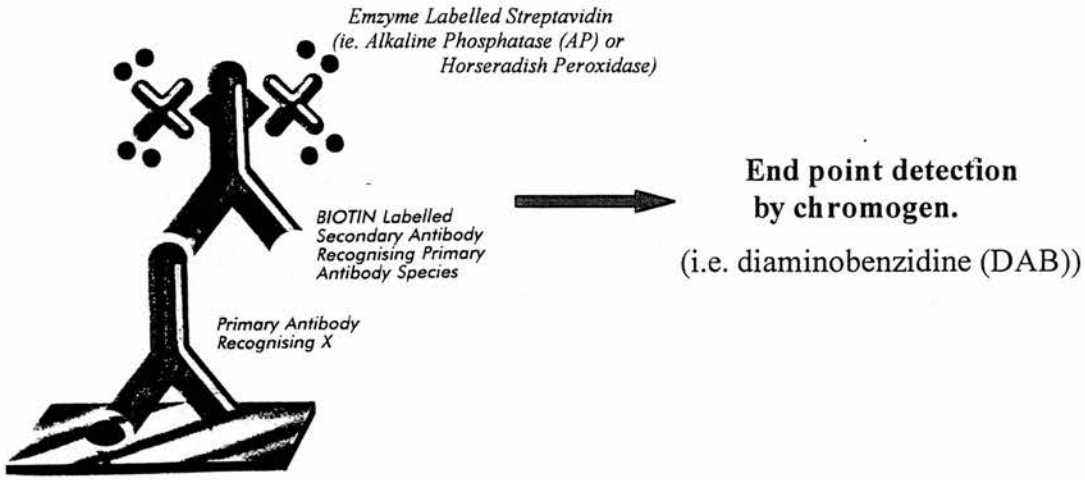
7. Addtion of ensyme labelled streptavidin (i.e. Alkaline Phosphatase or HRP) which bind to amplified biotin sites
8. Development of colour reaction by chromogen (i.e. DAB)

For fluoresence:

7. Addition of flurochrome lablled streptavidin.
8. Observed using fluroscope



(b) Avidin-Biotin complex technique (ABC)



(c) Immunofluorescence (IF)

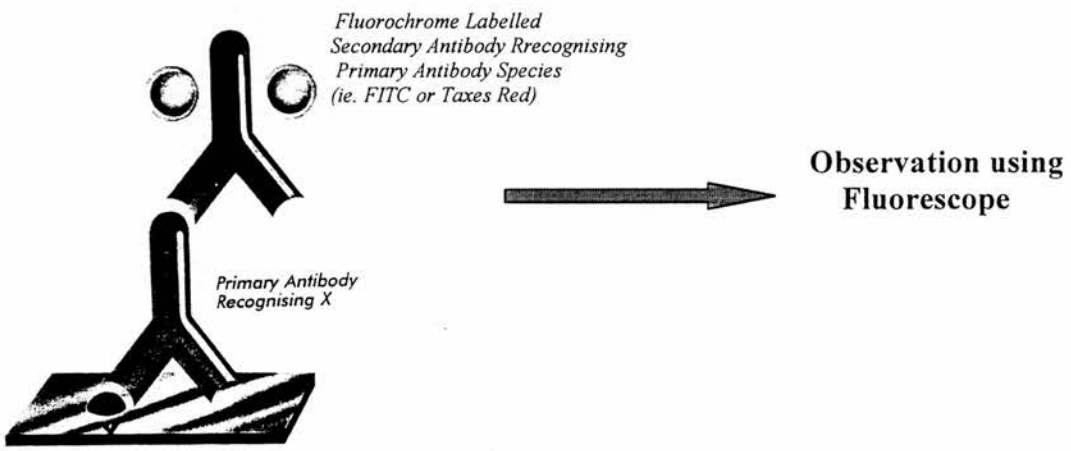


Figure 3. 1. Diagrammatic representations of staining techniques, Tyramide Signal Amplification techniques (a), Avidin-Biotin complex technique (b), and ordinary Immunofluoresences (c). Amplified signal in TSA technique is resulted from the increased biotin sites for enzyme labelled streptavidin.

This signal enhancement was first applied in the Enzyme-Linked ImmunoSorbent Assay (ELISA) (Bobrow *et al.,* 1989), and then in membrane immunoassays, such as Western or Southern blots (Bobrow *et al.,* 1991; Bobrow *et al.,* 1992), and finally introduced for histochemistry. The first attempt was made in Lectin immunohistochemistry (Adam, 1992) and resulted in a tremendous increase in sensitivity. Following this successful trial, CARD has now been adapted for many applications, including the amplification of in situ hybridization signal, and the detection of antigens using a primary or secondary biotin labelled antibody employing immunohistochemical or Western blotting (Berghorn *et al.,* 1994; Kerstens *et al.,* 1995; deHaas *et al.,* 1996).

No reliable data were available for the detection of HIV-1 antigens. However, it was hoped that the combination of antigen retrieval with optimized tyramide signal amplification would greatly enhance the results of HIV-1 immunohistochemistry.

In this study, one known HIV-1 p24 positive autopsy case was employed for optimising this signal-enhanced assay with DuPont anti-p24 antibody, which we termed tyramide signal amplification (TSA) technique. To examine the sensitivity and reproducibility of the TSA technique, this optimised signal enhancement was compared with the standard immunohistochemical Avidin-Biotin Complex (ABC) assay employing two commercially available primary antibodies, anti-P24 antibody (DuPont) and anti-gp41 antibody (Genetic systems), in a cohort HIV-1 positive autopsy cases using brain tissue. Subsequently, the applicability of the TSA technique was investigated for other antibodies including some specific cell markers, and with immunofluorescence (IF).

3.2 Modification and Optimisation of TSA Assay

A commercial DuPont NEN TSA kit was used to initiate the optimisation of anti-p24 antibody (DuPont). Known HIV-1 p24 positive frontal brain blocks taken from HIV encephalitis (HIVE) cases and processed with standard routine procedures

as previously described in section 2.2 were used as specimen cases. One cortical brain block from a normal HIV negative autopsy was included as a negative control. Serial sections were taken from each tissue block following the standard protocol (refer to section 2.5).

3.2.1 Optimisation of HIV-1 Anti-p24 Antibody

EXPERIMENTAL DESIGN. Both ABC and TSA methods were applied with serial dilutions of the primary mouse monoclonal anti-HIV-1 p24 antibody. The dilution of the secondary rabbit-anti-mouse biotinylabelled antibody (RAMBO) was fixed at 1 in 200. For the TSA assay, the streptavidin-HRP (SA-HRP) and biotinyl-tyramide reagent (BT) were applied following the instructions of the commercial TSA kit. The end point detection in both ABC and TSA techniques was carried out with the chromagen diaminobenzidene (DAB).

Tissue sections taken from a known HIV-1 negative normal cortex were exposed to exactly the same procedures as HIV-1 positive sections to serve as a negative control. In addition, one known p24 IHC positive section with omission of the primary antibody was also included as a negative control.

Moreover, to investigate the reproducibility of staining results, this set of experiments was repeated several times on different days with the same working conditions.

RESULTS. P24 positive cells were characterized by a strong cytoplasmic granular staining pattern. Staining results using serial dilutions of the anti-HIV-1 p24 mAb on serial sections are summarised in Table 3. 1. The staining results were graded by a self-defined system from 0 to 4, which was validated by sections examined blindly by at least three of a panel of individuals comprised of a technician, a collaborating post-doctoral researcher working in this field, a neuropathologist and myself. Score 0 represented no positive signal detected through the whole section. Score 2 represented the optimal ABC staining pattern as judged by long experience of the HIV-1 p24 ABC technique which was standard and reproducible in our lab,

and this standing pattern was used for comparative reference. Score 4 was given to a strongly positive staining pattern, which presented either more intense staining or more positive signals than the reference ABC section. If the staining intensity was lighter or the positive areas were decreased when compared with the reference ABC section, slides were graded 1. In contrast, slides were graded 3 when the staining intensity was slightly increased but the positive areas were similar with reference to the comparable ABC stained section.

Table 3. 1. Optimisation of anti-HIV-1 p24 antibody in serial sections using commercial purchased TSA kit and routine ABC technique parallel.

Dilution	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:51200
ABC	2	2	1	1	1	1	0	0	0
TSA	4	4	4	4	4	4	3	1	1

Key for Table 3. 1:
Staining results were read as --- **4**: strong positivity; **3**: moderate positivity; **2**: equal to optimal p24 ABC staining pattern; **1**: weakly positive; **0**: negative
Shaded area: dark background observed

Anti-HIV-1 p24 immunohistochemistry with microwave pretreatment using the TSA technique gave a greatly enhanced staining result when compared with the routine ABC technique. The optimal dilution of the anti-p24 mAb (DuPont) producing maximum performance was 1 in 3200 using the TSA and 1 in 200 using the ABC technique, which represents signal enhancement by a factor of 16. Moreover, the staining pattern detected by the TSA technique with a much lower concentration of anti-p24 mAb was even more intense and positive than the staining pattern of the ABC technique using higher concentration. At dilutions between 1 in 100 up to 1 in 1600 using the TSA technique, and 1 in 100 using the ABC technique, very high background staining was present. This dark background seriously interfered with the analysis of results, but was decreased at the higher dilutions described above as optimal for the two techniques. Generally, this background problem has been observed elsewhere with the increased sensitivity afforded by the biotin-tyramide amplification system (Bobrow *et al.,* 1992; Adam, 1992; deHaas *et*

al., 1996; von Wasielewski *et al.,* 1997). One probable reason is the presence of abundant endogenous biotin in tissues. This can be overcome by pretreating the tissue sections with a Vector biotin blocking kit. In fact, brain sections contain relatively little biotin and showed also less background than non-CNS tissues suggesting that endogenous biotin does bind reagents in the TSA technique leading to non-specific signal and high background. Other factors, including the concentrations of primary antibody, secondary antibody, streptavidin-HRP, biotinyl-tyramide, and the duration of incubation times, even the diluents, might also contribute to the increased background staining (Bobrow *et al.,* 1992; Adam, 1992; deHaas *et al.,* 1996; von Wasielewski *et al.,* 1997). Therefore, to eliminate the problem of severe background staining is a major task for the further optimisation of the TSA technique.

Negative controls, in which the primary antibody was omitted, and the normal, HIV-1 negative controls, showed no positive p24 signal with the ABC and TSA techniques. This confirmed the specificity of this anti-p24 mAb (DuPont) and showed that non-specific signals were not amplified when the TSA technique applied. All of the results were reproducible using the same techniques with the same conditions in our lab.

3.2.2 Optimisation of Streptavidin-HRP

EXPERIMENTAL DESIGN. Although the commercial DuPont NEN TSA kit was convenient, it was costly. Other reagents, including either in-house or singly purchased, were considered for use in order to replace the original reagents contained in the TSA kit. A single purchased reagent, streptavidin-horseradishperoxidase (SA-HRP) from the Dako Company, was used in order to replace the SA-HRP included in the DuPont NEN TSA kit. The SA-HRP reagent was used in two steps of the TSA procedure and the kit suggestion was to use it in a dilution of 1 in 500 diluted in TNB buffer for both steps. For the purpose of reducing background staining and seeking an optimal performance, different pairs of dilution of the Dako Streptavidin-HRP reagent were investigated in this optimisation. The conditions which were examined are described in Table 3. 2.

One section was stained using the complete set of the commercial NEN TSA kit, and was included as a positive control. Another section was stained using the ABC technique and this was also included as a basic reference. In addition, a section that was not exposed to the anti-HIV p24 mAb was required as a negative control.

RESULTS. The Dako SA-HRP reagent successfully replaced the SA-HRP reagent contained in the NEN TSA kit. The staining results are summarised at Table 3. 2. Again, the staining results were graded by a self-defined system from 0 to 4, which was validated by sections examined blindly by at least three of a panel of individuals comprised of a technician, a collaborating post-doctoral researcher working in this field, a neuropathologist and myself. Score 0 represented the pattern similar to the section following p24 ABC procedure, and score 4 represented the pattern either similar or stronger to the section using the complete set of the NEN TSA reagent. The scores 1 to 3 were given according to the positivity observed and compared to these two reference sections.

Table 3. 2. Results of the optimisation of streptAvidin-HRP reagent usage in TSA technique.

	Dilution							
1° StreptAvidin-HRP	100X	100X	100X	100X	250X	250X	**250X**	250X
2° StreptAvidin-HRP	100X	250X	500X	1000X	100X	250X	**500X**	1000X
Results	4	4	4	3	4	4	**4**	3
1° StreptAvidin-HRP	500X	**500X**	**500X**	500X	1000X	1000X	1000X	1000X
2° StreptAvidin-HRP	100X	**250X**	**500X**	1000X	100X	250X	500X	1000X
Results	4	**4**	**4**	2	2	1	0	0

Key for Table 3. 2:

a. The staining pattern of Anti-HIV-1 p24 diluted in 1:200 using ABC technique was scored " 0 " as a basic point of reference.

b. The staining pattern of Anti-HIV-1 p24 diluted in 1:3000 using the full commercial NEN TSA kit was scored " 4 " as a optimal point of reference.

c. Scores between 0 and 4 refer to the level of positivity observed.

d. Shaded areas: presence of background staining.

e. Bold character: the optimal staining pattern

The optimal performance was obtained when both SA-HRP (Dako) steps employed a 1 in 500 dilution in TNB buffer. At a dilution of 1 in 100 either at the first or second SA-HRP step, excessive background staining was observed. At a dilution of 1 in 1000 for the second SA-HRP step, weak enhancement was observed when the paired dilution of the first SA-HRP was 1 in 100 or 1 in 250. In contrast, at a dilution of 1 in 1000 for the first SA-HRP step, no signal enhancement occurred. The dilutions using 1 in 250 then 1: 500, or 1:500 then 1:250 gave a similar staining pattern as using 1 in 500 in both steps. For reasons of economy and convenience of working manoeuvres, it was concluded that the application of 1 in 500 dilution in both steps was the optimal protocol. Slight background staining was observed in some of these conditions. According to the literature (Bobrow *et al.,* 1992; Adam, 1992; deHaas *et al.,* 1996; von Wasielewski *et al.,* 1997), the use of biotinyl-tyramide itself may be a factor leading to background staining in addition to changing concentrations of primary antibody, secondary antibody and SA-HRP.

3.2.3 Optimisation of in-house Biotinyl-Tyramide

EXPERIMENTAL DESIGN. As considered above, the concentration of biotinyl-tyramide reagent, its diluent and the incubation time might contribute to the unpredictable background staining. In an attempt to reduce background staining and the cost of using the commercial DuPont NEN TSA kit while maintaining the advantage of increased sensitivity, in-house biotinylation of tyramide was undertaken according to the protocol published in Kerstens *et al.,* 1995. In this investigation, the dilutions of anti-p24 antibody, the secondary antibody, and SA-HRP (Dako) were carried out with the optimised conditions, but the dilution of in-house biotinyl-tyramide (BT) was varied. Also, several diluents for BT, including phosphate-buffer saline (PBS) containing 0.01% H_2O_2 (Kerstens *et al.,* 1995), Tris-buffered saline (TBS) containing 0.05% H_2O_2 (Macechko *et al.,* 1997), and 0.1 M Borate buffer pH 8.5 containing 0.01% H_2O_2 (Bobrow *et al.,* 1989) were examined. Moreover, the duration of BT incubation, which was recommended as 7 minutes in the commercial kit instruction, was varied from 1 minutes up to 15 minutes.

Sections were also stained using the commercial BT reagent and the diluent included in the NEN kit as reference positive controls and sections omitting the primary antibody were required as negative controls.

RESULTS. The maximal staining result was obtained when in-house biotinyl-tyramide reagent was diluted 1 in 200 with 0.1M Borate buffer pH 8.5 containing 0.01% H_2O_2. Compared with the biotinyl-tyramide contained in the NEN TSA kit, which was applied in a dilution of 1 in 50, in-house biotinyl-tyramide reagent gave a crisper and more condensed staining pattern. Surprisingly, the in-house BT reagent diluted in 0.1M Borate buffer pH 8.5 containing 0.01% H_2O_2 gave no background staining. This result was consistent and reproducible. Furthermore, long-term consistent results were obtained even when this homemade BT reagent was stored for 6 months at 4°C. The dilution of home-made BT needed to be re-optimised each time when making up a new batch of reagent.

Bringing these optimal conditions into the investigation of duration of BT incubation, the optimal results were obtained when sections were incubated for 4 minutes up to 8 minutes. When the section was incubated with BT reagent over 10 minutes, the staining pattern was blurred and background staining appeared. When the section was incubated with BT reagent for less than 3 minutes, little IHC enhancement was observed.

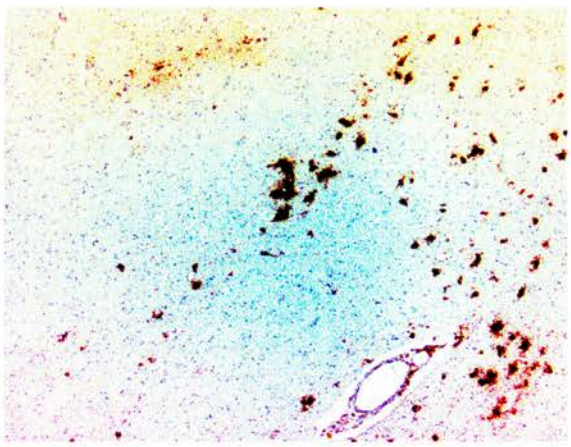
3.2.4 Short Summary of Applying TSA in p24 Detection

Thus far, the optimal conditions for the anti-HIV-1 p24 mAb (DuPont) TSA technique appeared to include a 1 in 3200 dilution for anti-HIV-1 p24 mAb (DuPont), 1 in 500 dilutions for both SA-HRP (Dako) steps, and in-house BT reagent diluted 1 in 200 in 0.1M Borate buffer pH 8.5 containing 0.01% H_2O_2 for 7 minutes.

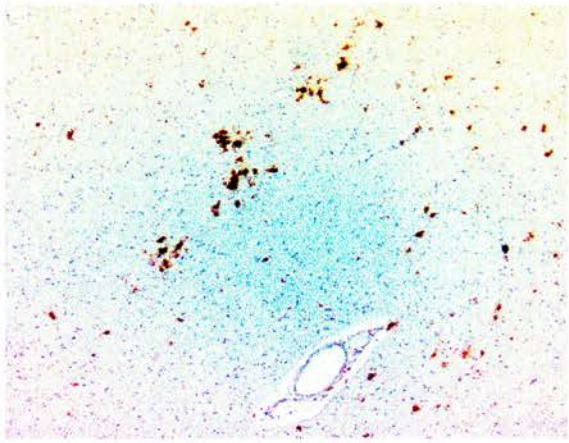
All of the optimisations were repeated several times on different days with the same working conditions and long-term consistent and reproducible IHC staining results were obtained.

Figure 3. 2. Detection of HIV-1 p24 antigen in serial sections of brain tissue from three cases (I, II and III) demonstrating increased sensitivity of antigen detection by TSA. The p24 antibody for TSA is 1 in 3200 and for ABC is 1:200. (DAB with haematoxylin counter stain; magnification, x25 for case I and II, x100 for case III).

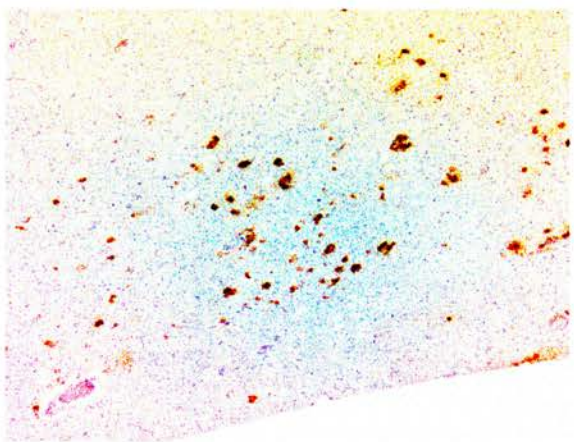
I-TSA



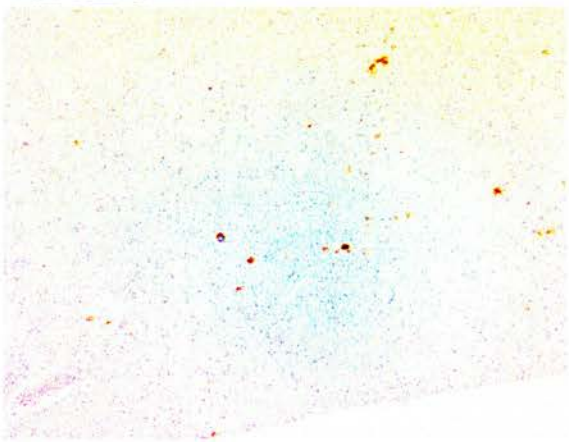
I-ABC



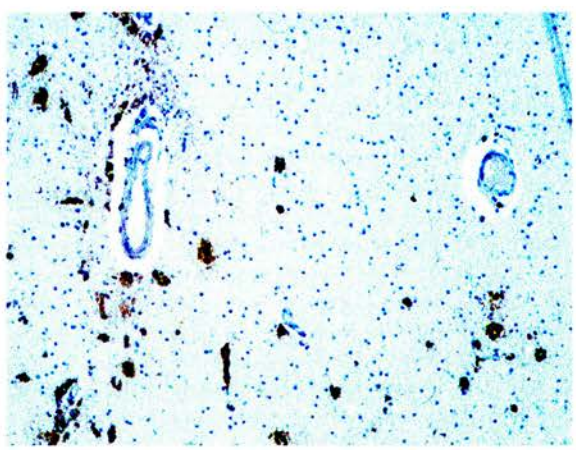
II-TSA



II-ABC



III-TSA



III-ABC

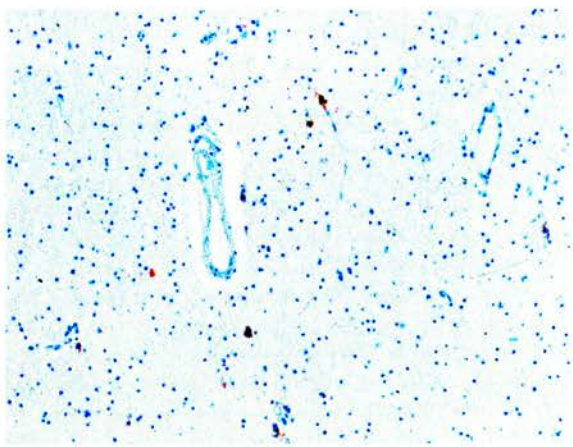


Fig. 3.2

3.3 Comparison Between the TSA and the ABC Methods

In order to compare the sensitivity of the ABC- and the TSA-IHC staining techniques, a comparison study was undertaken using serial sections of left frontal lobe brain tissue from cases described below. In addition to anti-HIV-1 p24 antibody, another mouse monoclonal antibody, anti-HIV-1 gp41 (Genetic Systems) was examined in parallel. This additional comparison using anti-HIV-1 gp41 mAb provided a further opportunity not only to compare the sensitivity between the ABC and the TSA technique, but also to investigate the applicability of the TSA method to other HIV antibodies.

TISSUE SAMPLES. The analysis was performed in frontal lobe tissue blocks taken from fifty randomly selected HIV-1 infected individuals, which have been described previously in Bell *et al.,* 1993 and Bell *et al.,* 1996a. These HIV-1 positive patients died either of AIDS-related illnesses or in the pre-symptomatic stage of infection. Tissues were processed following the standard procedure as described in sections 2.2 and 2.5.

EXPERIMENTAL DESIGNS. At least 6 serial sections were taken from each tissue block. Each set of slides was given the number 1 to 6, stained as negative control/ABC, p24 ABC, p24 TSA, gp41 TSA, gp41 ABC, negative control/TSA respectively. These immunohistochemical techniques were carried out with optimised conditions following the standard protocol (refer to chapter 2). The end point was visualized with diaminobenezidine (DAB) followed by haematoxylin counter stain.

Sections from a known HIV-1 positive tissue block were examined in parallel as positive controls. The first and sixth of each set of test slides were negative controls for the ABC and the TSA assays respectively. These controls were important for the confirmation of the staining results, because some of the tissue sections might non-specifically bind the secondary antibody or other reagents involved in the procedure.

3.3.1 Results of the Technical Comparison

3.3.1.1 Consistent Improvement in Staining Intensity

According to the observed intensity of staining pattern, the results of test sections were graded from 0 to 4. Score 0 representing negative, and 1 representing very sparse, to 4 representing very strongly positive signal observed in the whole section. Because of the likely different significance for pathogenesis, the intensity of antigen staining was assessed separately in white and grey matter (Table 3. 3 & Table 3. 4).

Table 3. 3. Summary of the results of the technical comparison

	Significant enhancement with TSA (sections)	Negative with ABC becomes weakly positive with TSA (sections)	Remains negative with TSA (sections)
Anti-p24 mAb	17	16	16
Anti-gp41 mAb	5	18	25

In comparison with the ABC method, the TSA technique demonstrated a reproducibly and considerably more intense staining pattern with both antibodies. As summarised in Table 3. 3, seventeen out of fifty cases showed consistently and significantly amplified signal in p24 antigen detection using the TSA technique. In another sixteen cases that were negative by the ABC technique, a weak but positive signal was demonstrated by the TSA technique. For anti-HIV-1 gp41 mAb (Genetic System), five cases were greatly enhanced and 18 cases, which were negative by ABC, showed weak positive signal with the TSA technique. The TSA technique also gave a considerable and reproducible amplification of the anti-gp41 mAb, however the performance of anti-gp41 mAb was not as good as the anti-p24 mAb in this trial. Within these fifty random cases, 34 showed positivity with anti-p24 TSA IHC but only 23 cases were detected by anti-gp41 TSA IHC. Moreover, in the cases, which were positive with both antibodies using TSA technique, the anti-p24 mAb mostly gave a stronger signal and showed a more localised pattern than the anti-gp41 mAb did.

Table 3. 4. The results of the comparison of ABC and TSA methods in detecting HIV-1 p24 and gp41 antibodies in a cohort of HIV-1-infected subjects.

NO	TSA-P24 WM	TSA-P24 GM	ABC-P24 WM	ABC-P24 GM	TSA-GP41 WM	TSA-GP41 GM	ABC-GP41 WM	ABC-GP41 GM
86-130	4	3	2	1	1	0	0	0
88-059	3	0	1	0	1	0	ND	
89-215	1	1	0	0	0	0	0	0
89-397	1	1	0	0	1	1	0	0
90-096	2	1	1	0	0	0	0	0
90-275	3	2	0	0	2	1	0	0
90-353	3	1	1	0	1	0	0	0
91-114	4	2	2	1	1	1	0	0
91-193	0	1	0	0	0	0	0	0
91-246	0	0	0	0	0	0	0	0
91-420	2	1	1	0	1	1	0	0
92-118	3	1	1	0	0	0	0	0
92-259	1	0	0	0	1	0	0	0
92-278	0	0	0	0	0	0	0	0
92-334	1	0	0	0	1	0	0	0
92-337	1	1	0	0	1	0	0	0
92-400	1	0	0	0	0	0	0	0
92-415	2	3	2	0	1	0	0	0
92-451	1	0	0	0	1	0	0	0
93-016	1	1	0	0	1	0	0	0
93-089	0	0	0	0	0	0	0	0
93-176	3	1	1	1	2	1	0	0
93-184	0	0	0	0	0	1	0	0
93-320	4	2	1	1	2	1	0	0
93-357	1	1	0	0	1	0	0	0
93-358-1	4	4	1	1	1	1	0	0
93-358-2	2	1	1	0	1	0	0	0
94-034	0	0	0	0	1	0	0	0
94-038	3	2	2	1	2	1	0	0
94-116	0	0	0	0	0	0	0	0
94-132	0	1	0	0	0	1	0	0
94-170	0	0	0	0	0	0	0	0
94-171	0	0	0	0	0	1	0	0
94-172	0	0	0	0	0	0	0	0
94-186	0	1	0	0	0	0	0	0
94-271	0	0	0	0	0	0	0	0
94-276	0	0	0	0	0	0	0	0
94-277	ND		0	0	0	0	0	0
94-280	0	0	0	0	0	0	0	0
94-284	0	0	0	0	0	0	0	0
94-313	0	1	0	0	0	0	0	0
95-004	3	3	1	1	0	0	0	0
95-010	0	0	0	0	0	0	0	0
95-024	2	1	0	0	1	1	0	0
95-040	0	0	0	0	0	0	0	0
95-043	1	0	0	0	0	0	0	0
95-079	1	0	0	0	0	0	0	0
95-088	1	0	0	0	0	0	0	0
95-108	1	1	0	0	0	0	0	0
95-284	2	2	1	1	2	2	1	1

Key for table 3.4

Intensity of staining score 4: very strong; 3: strong; 2: moderate; 1: weak; 0: no staining

WM: white matter; GM: grey matter

For those sections showing p24 or gp41 positivity, the enhanced signal was

observed both in WM and GM. No significant differences of enhancing capability were found in these two areas.

3.3.1.2 Improvement in Staining Sensitivity

To assess whether the TSA method resulted in an increase in number of p24 positive foci when compared to the ABC method, eight cases were selected for quantitation. The number of antigen positive foci was counted within a 4 X 3 inch recticle (Olympus) in two separated areas of the tissue section and by two individuals (Dr. Strappe and myself) (Table 3. 5). To ensure that the same area was examined in each pair of serial sections, specific fields were selected either near the edge of the section or related to a blood vessel.

In addition to increasing the staining intensity, the results in this trial demonstrated an increased number of p24 positive foci with the application of the TSA method. No positive signal was observed in the two negative control slides, No.1 and No.6 in each series suggesting that the signal amplification was specific. Moreover, the additional positive cells detected by the TSA technique were morphologically microglial cells or macrophages, which are known to be the major targets of HIV-1 in brain tissue. These findings provided the evidence confirming the specificity of technical amplification with the TSA method. These findings have been published in Strappe *et al.,* 1997.

Table 3. 5. Comparison of the number of p24 antigen positive foci detected by TSA and ABC methods on serial sections of brain tissue from eight study subjects.

Patient No.	TSA	ABC
93-176	20	14
	28	18
92-118	28	18
	32	22
93-358	17	10
	20	10
91-114	34	26
	34	15
86-130	32	26
	34	20
88-059	25	11
	21	09
90-353	22	12
	14	08
90-096	47	31
	33	17

Two areas of each section were examined within a defined recticle

Wilcoxon Signed Ranks Test:

- **Ranks**

	N	Mean Rank	Sum of Ranks
TSA - ABC Negative Ranks	0[a]	.00	.00
Positive Ranks	***16[b]***	***8.50***	***136.00***
Ties	0[c]		
Total	16		

a TSA < ABC; b TSA > ABC; c ABC = TSA

- **Test Statistics[b]**

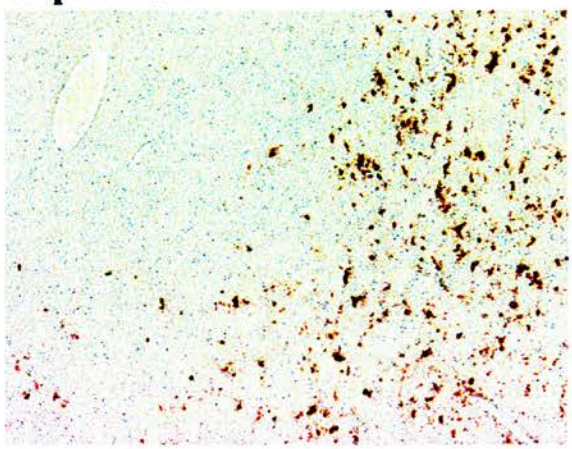
	TSA - ABC
Z[a]	-3.532
Asymp. Sig. (2-tailed)	.000

a Based on negative ranks. Results of TSA method are significant increased.

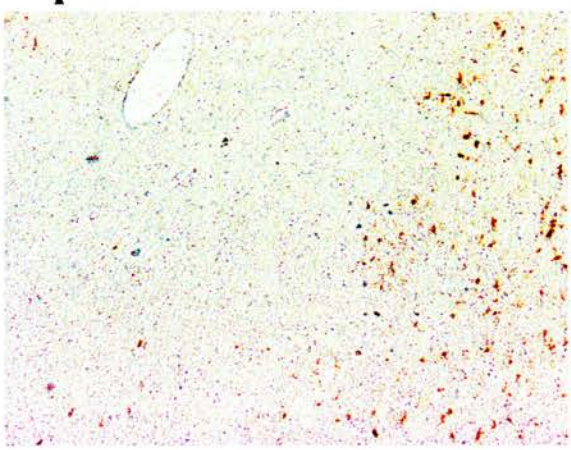
b Wilcoxon Signed Ranks Test

Figure 3. 3. Comparison of ABC and TSA staining techniques for HIV-1 p24 and gp41 antibodies in serial sections of brain tissue from two cases A and B demonstrating increased sensitivity of antigen detection by TSA, and the better labelling ability of p24 mAb. The dilution of p24 mAb is 3200 for TSA and 200 for ABC, and of gp41 mAb is 2000 for TSA and 100 for ABC. (DAB with haematoxylin counter stain; magnification, x25 for case A, x100 for case B).

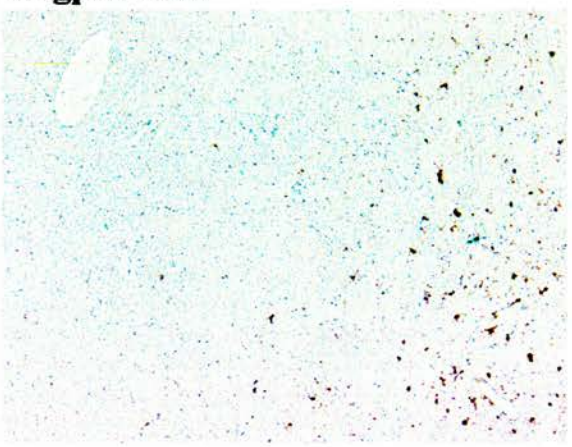
A-p24-TSA



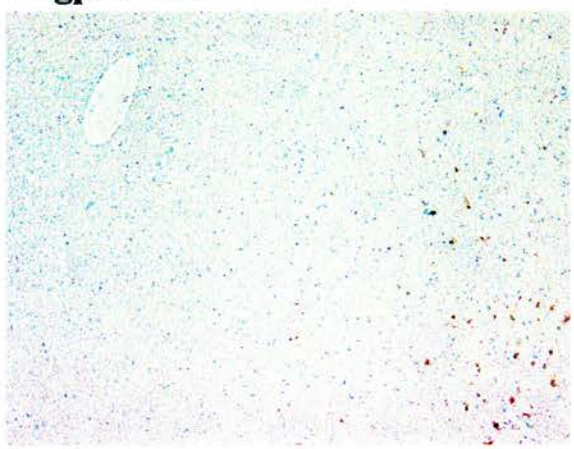
A-p24-ABC



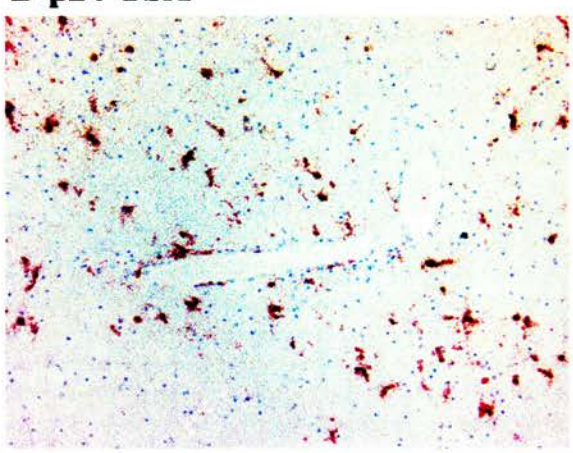
A-gp41-TSA



A-gp41-ABC



B-p24-TSA



B-gp41-TSA

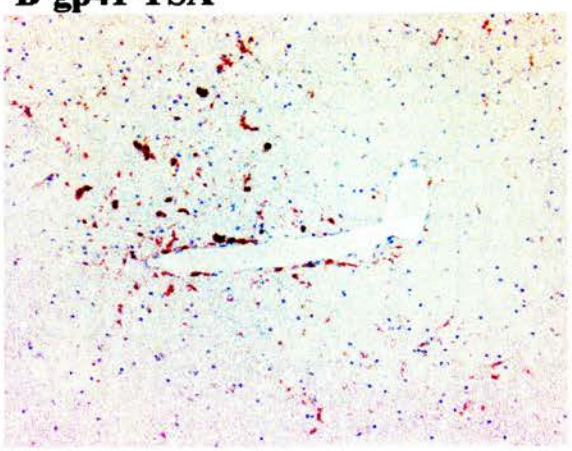


Fig. 3.3

3.4 Applications of TSA Signal Enhancing System

3.4.1 TSA in Various Cell Markers Staining

To evaluate the effects of the TSA technique with other antibodies, which might be used for further cellular identification, immunohistochemistry was applied using the TSA and routine ABC techniques for certain cell markers, including T cell markers (CD3, CD8, MT1 and UCHL1), B cell markers (L26 and MB1), dendritic cell marker (CD21), macrophage marker (PG-M1) and the astrocyte marker (GFAP).

EXPERIMENTAL DESIGNS. Routinely fixed paraffin-embedded positive control tissues for each antibody examined in this trial were processed according to the protocol of section 2.3. The TSA and the ABC techniques were performed according to the protocols of section 2.5, and end point detection was carried out with chromogen diaminobenzidene (DAB). For each primary antibody, at least six different dilutions were examined. If the results were not conclusive, further dilutions were examined.

For comparison of detection methods, the slides for a given marker using the ABC and the TSA technique were carried out on the same day using identical solutions, detection system, and substrate (DAB). Therefore, any variations caused by factors other than the use or omission of TSA could be excluded.

RESULT. The dilution giving the best performance for each antibody is listed in Table 3. 6. All of these results were reproducible and consistent.

Generally, using the TSA amplified system allowed at least eight fold higher dilution of primary antibodies in colour detection, but no extra positive signal was observed when comparing the optimal ABC slides with the optimal TSA slides in these antibodies. As listed in table 3.8, the dilution for the GFAP was dramatically raised from 1 in 2000 for the routine ABC up to 1 in 32000 after the TSA method was applied. And for the L26 antibody, the dilution was raised from 1 in 500 using the ABC method up to 1 in 16000 using the TSA method, which was enhanced by a

factor of 32. Moreover, for the antibodies, anti-CD8 mAb and MB1, which only worked routinely with a very high concentration, the TSA technique showed much improved performance using a lower concentration.

The staining intensity was improved consistently for most of the antibodies, excluding GFAP, CD3 and UCHL1. For the anti-CD8 mAb and PG-M1, the staining pattern with the TSA technique was much more intense and crisper compared with the ABC technique. But for the anti-CD3 polyclonal Ab, GFAP and UCHL1, although the positive signal could be detected with very low concentration, the staining patterns with the TSA were slightly blurred and less sharp compared with the ABC technique.

Moreover, background staining was observed with some of the cell markers, such as L26, MT1 and UCHL1, and especially for anti-CD3 antibody and GFAP with the TSA technique. This background problem could not be solved by using Vector Biotin blocking kit. It was only reduced when a higher dilution was applied; however the positivity of the staining signal was decreased at the same time.

Table 3. 6. Optimal dilutions of each antibody using the ABC and the TSA detection systems

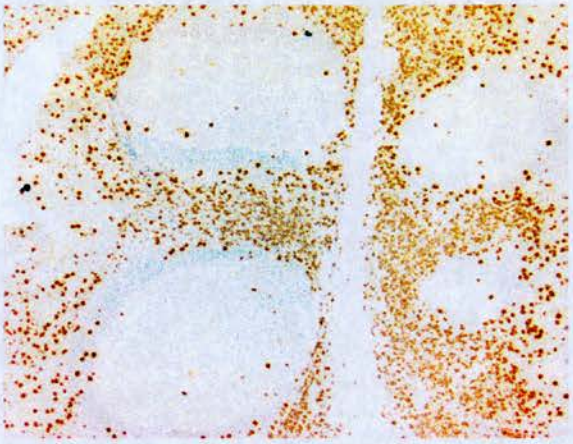
Primary antibody	ABC	TSA	Conventional IF	TSA-IF
P24	1:200	1:3200	N/A	1:150
CD3	1:150	1:1200	1:25	1:150
CD8	1:50	1:800	N/A	1:100
CD21	1:200	1:1600	1:100	1:400
PG-M1	1:200	1:6400	1:50	1:200
GFAP	1:2000	1:32000	1:250	1:2000
L26 (CD20)	1:500	1:16000	1:100	1:500
MB1	1:50	1:800	N/A	1:75
MT1	1:150	1:2400	1:25	1:150
UCHL 1	1:350	1:5600	1:25	1:300

N/A: undetectable with dilution 1:25.

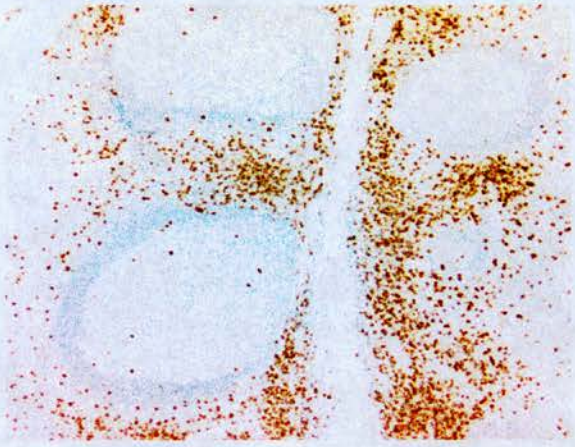
Figure 3. 4. Comparison of ABC and TSA techniques in cell surface markers staining. TSA technique demonstrated an intense staining signal in CD8, PGM1, and CD21. (DAB with haematoxylin counter stain; magnification, x50).

- Dilutions of each antibody:
  CD8-TSA: 1:800; CD8-ABC: 1:50 (in serial sections of tonsil)
  PGM1-TSA: 1:6400; PGM1-ABC: 1:200 (in serial sections of brain tissue)
  CD21-TSA: 1:1600; CD21-ABC: 1:200 (in serial sections of lymph node)

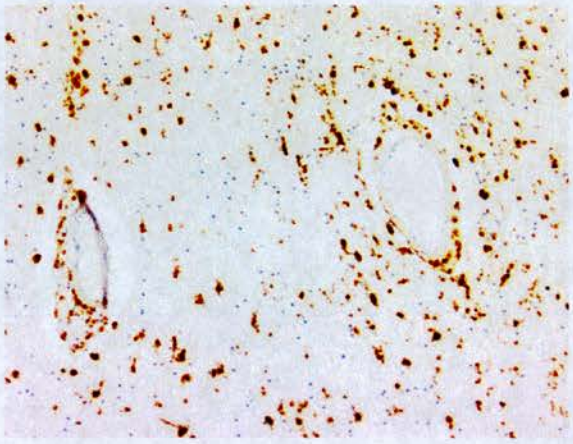
CD8-TSA



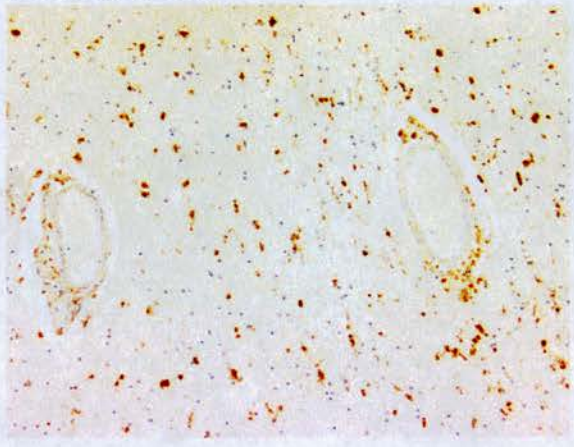
CD8-ABC



PGM1-TSA



PGM1-ABC



CD21-TSA

CD21-ABC

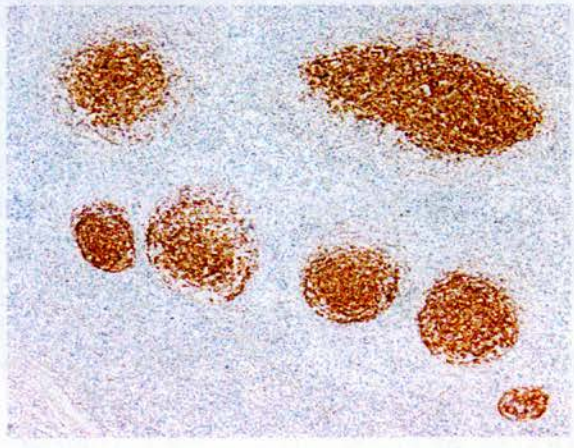


Fig. 3.4

3.4.2 TSA in Immunofluorescence

Immunofluorescence (IF) has been reported as a useful tool in multiple-labeling detection for demonstrating the cellular localisation of viral proteins. However, the detection sensitivity has seriously limited its application, especially in formalin-fixed paraffin-embedded tissues. The optimising investigations described above suggested that application of the TSA technique could assist in enhancing the staining sensitivity. Therefore, to evaluate the effects of applying the TSA technique in immunofluorescence, Texas red-conjugated streptavidin (SA-Texas Red) was employed to replaced the second SA-HRP(refer to Figure 3. 1), and as an end-point labeling which was then visualised by the fluorescence microscopy.

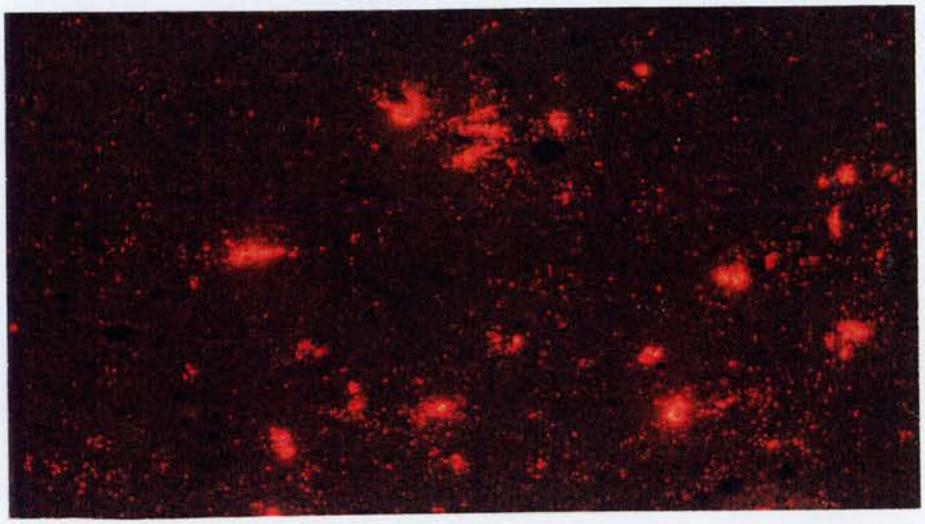
EXPERIMENTAL DESIGNS. The TSA-IF and the conventional IF techniques for anti-p24 mAb and a variety of cell markers, including T cell markers (CD3, CD8, MT1 and UCHL1), B cell markers (L26 and MB1), dendritic cell marker (CD21), macrophage marker (PG-M1) and astrocyte marker (GFAP) were carried out according to the protocols of section 2.5. For comparison of detection methods, the staining using a given marker and using the conventional IF and the TSA-IF technique was carried out on the same day using identical solutions, and detection system. Therefore, any variations caused by factors other than the use or omission of TSA could be excluded. For each primary antibody, at least six different dilutions were examined. If the results were not conclusive, further dilutions were tested.

RESULT. The dilution giving the best performance for each antibody is listed in Table 3. 6. All of these results were reproducible and consistent.

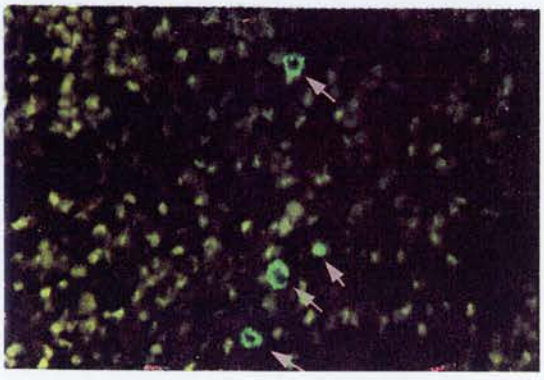
The application of TSA in immunofluorescence again demonstrated a great enhancement. In general, the TSA-immunofluorescence (TSA-IF) allowed the use of at least a four fold higher dilution of primary antibodies in fluorescence detection, and the staining pattern using TSA-IF was much clearer and cleaner than using conventional immunofluorescence. Commonly, the optimal dilution used for a conventional immunofluorescence technique was very concentrated. For the

antibodies, anti-p24, CD8 and MB1, signal was undetectable even with a concentration of 1 in 25. The use of such high concentration was associated with background staining and interfered with analysis of results. With the TSA-IF, because lower concentration of antibody was used, the background problem was curtailed. In this trial, background staining with the TSA-IF was only observed for GFAP. However, since the positivity of its staining signal was very strong, in contrast the background staining was acceptable in this case.

(a) HIV-1 p24 TSA-IF



(b) CD8-TSA-IF



(c) PGM1-TSA-IF

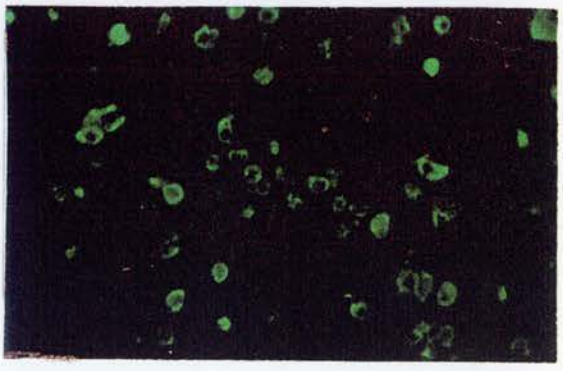


Figure 3. 5. TSA-Fluorescence in p24 (a), CD8 (b) and PGM1 (c) detection demonstrating clear background and intense signal. (P24 was labelled with SA-Texas Red. CD8 and PGM1 were labelled with SA-FITC; magnification, x200).

3.5 Discussion

3.5.1 Enhancement of TSA Technique in HIV-1 Antigen Detection

Immunohistochemistry (IHC) has been widely used in diagnostic and investigative histology and pathology. It is indubitable that IHC provides valuable cellular information directly linked to cytopathology. Due to the limitations of antigen masking and the relative insensitivity of staining techniques, its application in HIV-1 research fields has been relatively restricted, especially in the study of paraffin-embedded samples of non-CNS tissues. Only partial success has been reported previously using enzymatic antigen retrieval and overnight incubation with monoclonal antibodies to HIV-1 gag p24 antigen or envelope gp41 antigen in certain cryostat and paraffin embedded tissues (Chad *et al.,* 1990; Kure *et al.,* 1990; Esiri *et al.,* 1991; Martin *et al.,* 1992). When the antigen retrieval technique of microwave irradiation was developed recently the sensitivity of immunohistochemical staining was increased because of its great potential to solve some problems of antigen masking (McQuaid *et al.,* 1995). However, it was clearly not ideal for the immunohistochemical detection of HIV-1 viral antigens, in that PCR positive tissues often remained IHC negative.

In this investigation, the biotin-tyramide amplification system (TSA) combined with microwave antigen retrieval was employed for the first time in the detection of HIV-1 viral proteins in formalin-fixed paraffin-embedded tissue sections. The protocol, which was optimised in this study, gave a consistent and considerable enhancement in the detection of HIV-1 viral antigens, especially for anti-p24 mAb. Compared with the routine ABC method, the p24 TSA IHC constantly gave a more intense and crisp staining pattern, and the most exciting finding was that the TSA technique revealed more positive signals indicating that productive infection was more widespread than previously detected. In addition, a much lower concentration of anti-p24 mAb proved to be of a great benefit in economical use of research antibodies. For instance the DuPont HIV-1 p24 antibody was withdrawn during the progress of this research despite being a satisfactory antibody for commercial use. Use of the TSA technique has allowed the continued use of reserve stocks of antibody.

With the biotin detecting system, background staining was usually associated with the increased intensity. Surprisingly, with optimal conditions, the TSA technique demonstrated a clear background in the p24 and gp41 IHC staining. Moreover, no staining was shown in sections in which the primary antibody was omitted, and the normal HIV-1 negative cortex control. This evidence confirmed that the amplification via tyramine detection was specific.

When the TSA technique was utilised with the two currently reliable HIV-1 antibodies, anti-gp41 TSA IHC did not perform as well as anti-p24 TSA IHC. Much higher dilution was possible (from 1 in 100 using the ABC technique to 1 in 2000 using the TSA technique), and the results obtained in the technical comparison study (section 3.3) showed that more cases presented positive gp41 signal after the TSA technique was applied, and the intensity of gp41-staining pattern was also improved. However, carefully comparing the staining results of gp41 with p24 in the same case, p24 TSA IHC gave a consistently more intense and localised staining than gp41 TSA IHC did. In the previous HIV-1 IHC studies, the anti-gp41 mAb (Genetic System) was frequently employed, which works consistently with a routine ABC technique, but sometimes binds non-specifically to macrophages (Sinclair & Scaravilli, 1992) and an irrelevant epitope in Alzheimer neurofibrillary tangles (Kure *et al.*, 1990; Morgello *et al.*, 1998). The anti-p24 mAb (DuPont) was relatively specific (Kure *et al.*, 1990; Sinclair & Scaravilli, 1992), and was considered to be associated with disease progress being a good prognostic marker (Allain *et al.*, 1987; Sei *et al.*, 1989; Fahey *et al.*, 1990). However its application was restricted by the complicated staining protocol and inconsistent results (Chad *et al.*, 1990; Kure *et al.*, 1990). But now, according to the present results, when combined with the microwave irradiated pretreatment, p24 TSA IHC gave superior staining results for viral proteins and was the best choice for screening paraffin sections in HIV-1 IHC studies.

3.5.2 Application of TSA Technique with Other Cell Marker Antibodies

The TSA technique gave an added benefit for the anti-p24 mAb IHC in paraffin-embedded tissues, however the advantages of using TSA technique for other antibodies examined in section 3.2.3 were limited.

There is no doubt that the TSA technique consistently allowed a considerable reduction in primary antibody concentration with all these cell markers. However the improvement in staining intensity was variable. Some antibodies, such as the anti-CD8 mAb and PG-M1 showed more intense staining patterns with the TSA technique, while anti-CD3 polyclonal Ab and GFAP showed less well-localised staining patterns. The differences in enhancement between the different antibodies were difficult to explain. Possibly, the composition of the surrounding tissue may affect the deposition of activated tyramine because this substance is known to react with electron-rich moieties on the tissue surface (Bobrow *et al.,* 1989; von Wasielewski *et al.,* 1997). Thus, each antibody requires individual modifications for achieving optimal results. The conclusion in this study was that if results with the primary antibody are inconsistent using a routine technique, such as with anti-p24 mAb, and anti-CD8 mAb, then TSA was effective in improving the staining results both in reducing antibody concentration and in improving staining intensity. However, for cell markers such as anti-CD3 and GFAP which gave good and consistent results with routine IHC, the advantage of using the TSA technique was solely in using higher dilutions. Coincidentally, anti-CD3 and GFAP are polyclonal antibodies, which are generally less specific and purified. Whether the relatively small improvement for these two antibodies implies that the TSA technique is unsuitable for use with polyclonal antibodies cannot be deduced from the number of cases provided in this study, but for future studies, these cell markers will not be combined with TSA IHC unless a better protocol is developed.

3.5.3 Advantages of Applying TSA Technique in Immunofluoresence

An exciting parallel finding was the significant improvement of applying TSA in the immunofluoresence procedure. The TSA-immunofluorescence (TSA-IF) method performed much better than the conventional immunofluorescence technique. It allowed not only the use of higher dilutions, but also gave much clearer staining patterns than conventional immunofluorescence. Previously the conventional IF technique for p24 antigen detection using a fluorochrome labelled secondary antibody was insensitive even applying a very high concentration of p24 antibody (1 in 5). The TSA immunofluorescence technique for p24 antigen detection gave an intense fluorescence signal with no background staining and allowed the use of a lower dilution of 1 in 150. Such improvement was also found in the TSA-IF detection of cell markers. The ability of this tyramine amplification system to utilise a lower concentration of primary antibody efficiently diminished the non-specific signal, which was usually associated with higher concentration of primary antibody required for conventional IF. A much clearer background coupled with more intensive signal gave evidence of the optimal fluorescent performance. This benefit of using TSA-IF provides an opportunity for the development of double-labelling immunofluorescence (Wang *et al.,* 1999a), which would be useful for the identification of the cell type that is infected with HIV-1.

3.6 Summary

The TSA technique is an important step in the developmental progression of IHC, especially in detection of HIV-1 p24 antigen. Since the working conditions had been optimised, the sensitivity of detection was greatly enhanced, and the TSA technique was considered the best tool for the p24 screening investigation in various organs.

Chapter 4: Cell and Tissue Distribution of HIV-1 in Different Stages of Infection

4.1 Introduction

While several new insights in various fields of HIV research have been gained, especially over the last three years, a better understanding of HIV pathogenesis and the mechanisms underlying HIV-1 related disease in specific tissues such as the brain is still a high priority in tracking the medical impact of the pandemic.

Identification of the cellular host range of HIV-1 is important for understanding the pathogenesis. Originally, the CD4+ lymphocytes were recognised as a major target for HIV infection, and the destruction of CD4+ lymphocytes by HIV-1 was thought to be the major cause of the observed pathological changes (reviewed in Levy, 1998). Recently, according to the improved techniques, such as using the PCR detection with extracted tissues or individual cells, or electron microscopy, or in situ hybridisation, HIV-1 proviral DNA or virus particles have been found in several tissues, including cells of the haematopoietic lineage (Schnittman *et al.,* 1989; Bagasra *et al.,* 1992; Bagasra *et al.,* 1993; Bagasra & Pomerantz, 1993; Embretson *et al.,* 1993a; Embretson *et al.,* 1993b; Patterson *et al.,* 1993; Donaldson *et al.,* 1994a; Nuovo *et al.,* 1994b; Livingstone *et al.,* 1996), brain (Wiley *et al.,* 1986; Bell *et al.,* 1993; Donaldson *et al.,* 1994a; Nuovo *et al.,* 1994a; Bagasra *et al.,* 1996), gastrointestinal tissue (Fox *et al.,* 1989; Kotler *et al.,* 1991; Gill *et al.,* 1992; Kotler *et al.,* 1993; Donaldson *et al.,* 1994a), lung (Donaldson *et al.,* 1994a; Dolei *et al.,* 1996; Huang & Stansell, 1996), and others (Tschachler *et al.,* 1987; Nuovo *et al.,* 1993; Bagasra *et al.,* 1994; Qureshi *et al.,* 1995; Uittenbogaart *et al.,* 1996), albeit at a very low level in some (reviewed in Levy, 1998). Also, using cell culture systems, a wide variety of human cell lines have been demonstrated to be susceptible for HIV-1 infection, including tissue macrophages, NK cells, dendritic cells and other CD4-negative cell lines (reviewed in Levy, 1998). These observations implied the polytropic nature of this virus, and revealed a wider range of possibilities for virus to be concealed in different tissues.

Macrophages are CD4+ migratory cells derive from bone marrow precursors. They are found in most tissues of the body, and have been found to propagate the HIV-1 infection (reviewed in Levy, 1998). These large mononuclear phagocytic cells are important in innate immunity, in early non-adaptive phases of host defense, as antigen-presenting cells, and as effector cells in humoral and cell-mediated immunity (Janeway & Travers, 1996). Generally, infection of macrophages is associated with low-level production of virus, and the HIV-1 virion is found sequestered in intracellular vacuoles (Gendelman *et al.,* 1989). It is postulated that macrophages might be the first cells infected at the mucosal barrier in the case of sexual transmission and that infection of this cell type could be responsible for the systemic dissemination of the virus found during primary infection (Levy, 1998). The observation of infected macrophages in lamina propria has provided evidence of HIV-1 infection in the intestinal system (Kotler *et al.,* 1991; Kotler *et al.,* 1993). Moreover, cells of the monocyte/macrophage lineage in the central nervous system, including microglial cells, multinucleated giant cells (MGCs) and brain macrophages have also been found to be the major targets of HIV-1 infection, which can be demonstrated by immunohistochemical staining (reviewed in Bell, 1998). However in lymphoid tissues, relatively small numbers of infected macrophages were detected (Embretson *et al.,* 1993a; Chun *et al.,* 1997).

Sharing a stem cell precursor origin with macrophages, dendritic cells (DC) are specialised antigen-presenting cells that are widely distributed in the body except in the CNS, and which are named Langerhans cells in the skin, vagina and cervix, interdigitary cells in the LNs, dendritic cells (DC) in thymus, interstitial DC in the heart, lungs and intestine, and blood-derived DC in the peripheral circulation (Janeway & Travers, 1996). Certain of these related cells appear to be susceptible to HIV infection (Tschachler *et al.,* 1987; Cameron *et al.,* 1996; Knight, 1996; Knight & Patterson, 1996). Monocyte-derived cultured DC and DC obtained directly from blood have been shown to be susceptible for HIV-1 infection in cell culture (Tsunetsugu-Yokota *et al.,* 1995; reviewed in Knight, 1996). Weissman *et al.* demonstrated that one subpopulation of DC was susceptible to infection with HIV-1 *in vitro* (Weissman *et al.,* 1995). HIV-1 proviral DNA and mRNA have

been detected previously in Langerhans cells from skin of infected patients (Dusserre *et al.,* 1992). Their presence in genital mucosa identified them as potentially the primary target cell infected by HIV-1 in heterosexual transmission (Hussain & Lehner, 1995; Soto-Ramirez *et al.,* 1996), and there might be a specific tropism of HIV-1 for Langerhans cells that correlates with the geographical distribution of the HIV isolates and with the HIV-1 subtype (Soto-Ramirez *et al.,* 1996). Some investigators suggested that a small portion of DC in various tissues of the body could be productively infected (Dusserre *et al.,* 1992; Weissman *et al.,* 1995; Knight, 1996), However others suggested that DC could not be infected but might transfer bound virus particles to T lymphocytes in the blood and lymphoid tissue (Cameron *et al.,* 1996).

HIV-1 proviral DNA has been found frequently in LN (Pantaleo *et al.,* 1991; Embretson *et al.,* 1993a; Embretson *et al.,* 1993b; Pantaleo *et al.,* 1993a; Pantaleo *et al.,* 1994). Histological and electron microscopic examinations have indicated that in the early stages of HIV-1 infection, large amounts of viral particles can be visualized in the villous processes of follicle dendritic cells (FDC) in the germinal centres of LNs (Pantaleo *et al.,* 1991; Spiegel *et al.,* 1992; Pantaleo *et al.,* 1993a). FDC is a specialised cell type within the lymphoid follicles, which are mainly constructed of a dense network of closely packed B cells forming the germinal centre, and which function to promote the maturation of the humoral immune response (Knight, 1996; Janeway & Travers, 1996). Unrelated to other types of the dendritic cells, the cellular origins of these branched FDC are obscure. They are not bone marrow derived but probably mesenchymal (Knight, 1996; Janeway & Travers, 1996). Whether the large number of viral particles observed in association with FDC processes reflects their infectability or merely represent HIV-1 trapped on the surface of these cells is not known. Some groups failed to detect productive HIV-1 infection of the FDC (Embretson *et al.,* 1993a; Pantaleo *et al.,* 1993a; Reinhart *et al.,* 1997), while other investigators reported an average of 20% of these CD21+ cells showing HIV-1 proviral DNA (Stahmer *et al.,* 1991; Spiegel *et al.,* 1992).

Other cells, including astrocytes, oligodendrocytes, neuron, goblet cells, columnar epithelial and enterochromaffin cells, have shown susceptibility for HIV-1 infection *in vitro*, although they are CD4-negative (Gill *et al.,* 1992; Moses *et al.,* 1993; Nuovo *et al.,* 1994a; Albright *et al.,* 1996; Strappe *et al.,* 1998). Similarly, mature cytotoxic T cells (CTLs) do not express surface CD4 molecule, yet infection of CD8+ T lymphocytes in the peripheral circulation and in lung has been demonstrated (Sadat-Sowti *et al.,* 1994; Bofill *et al.,* 1995; Semenzato *et al.,* 1995; Livingston *et al.,* 1996; Yang *et al.,* 1998). In general, the mechanisms of HIV-1 infection of these CD4-negative cells remain speculative. HIV-1 may infect cells through a different receptor or, in the case of CD8-positive lymphocytes, infection may occur during maturation or on antigenic stimulation where the CD4 molecule may be transiently expressed (Flamand *et al.,* 1998; Yang *et al.,* 1998).

Previous studies had postulated that the CD4 molecule expressed alone was not sufficient for HIV-1 infection (Maddon *et al.,* 1986; Li *et al.,* 1990; Chesebro *et al.,* 1990; Cao *et al.,* 1990). A number of proteins from a superfamily of G-protein-coupled seven-transmembrane-domain chemokine receptors were found to be co-factors required for HIV-1 entry (Alkhatib *et al.,* 1996; Berson *et al.,* 1996; Deng *et al.,* 1996; Dragic *et al.,* 1996; Feng *et al.,* 1996; Kozak *et al.,* 1997). Generally, NSI/macrophage tropic HIV variants use the β-chemokine receptor (CCR5) and SI/lymphotropic variants use the α-chemokine receptor (CXCR4) (refer to section 1.3.1.2). In 1996, Paxton *et al.* reported that a small proportion of the population who expressed defective CCR5 receptors were highly resistant to infection with HIV-1 even after repeated exposure through sexual intercourse with HIV-positive partners (Paxton *et al.,* 1996). How these co-receptors contributed to cytopathogenicity and disease progress is not yet clear. It has been found that various human cells could only be infected with specific HIV-1 variants, and different HIV-1 strains retain different cytopathogenicity and replicating abilities in specific human cells. The differences in *in vivo* cellular tropism have been hypothesised to be controlled by the usage of co-receptors (Dragic *et al.,* 1996; Kozak *et al.,* 1997).

The exact interaction between HIV-1 and different cell types is poorly understood. It was believed that direct and productive infection of these tissues *in vivo* might be responsible for the clinical abnormalities observed in HIV-1-infected individuals. This part of the study was aimed to investigate the cellular distribution and localisation of HIV-1 infection in various tissues. As discussed in chapter 3, the IHC staining assay can provide valuable cellular information directly linked to cytopathology, especially within formalin-fixed paraffin-embedded tissue samples. However the application in HIV studies was apparently limited by insensitivity of the technique. In order to determine the distribution and localisation of the HIV-1 infection *in vivo*, a improved technique for HIV-1 immunohistochemistry has been established, namely the tyraminde-signal amplification (TSA) system. According to the results shown in the previous sections (refer to chapter 3), the TSA technique showed better performance than the ABC technique in HIV-1 immunohistochemistry, especially with the anti-HIV-1 p24 antibody (DuPont) in our lab. Moreover, a few cases in the comparison trial (refer to section 3.3), which showed negative staining with the ABC method became weakly positive with the TSA method. Therefore, it was considered potentially valuable to apply the TSA technique to a more extensive set of organs and tissues for which HIV-1 immunostaining had been previously unsuccessful. Also, the general distribution of the co-receptors CXCR4 and CCR5 was investigated in these tissues using the ABC technique.

4.2 Study Subjects

Study cases were obtained from the MRC funded brain bank in Edinburgh as described in section 2.1. Fifty-three patients were selected randomly for this trial, including 43 patients who died of AIDS-related illness and 10 patients who died before the onset of AIDS. Tissue blocks were taken, if available, from colon, ileum, kidney, liver, LN, left frontal lobe of brain, lung, spleen, and thymus from each study subject, and processed following the standard procedure as described in section 2.2. The selection of tissue blocks was more restricted in pre-AIDS cases

which were generally fiscal autopsies. Clinical information for each patient is briefly summarised in Table 4. 2.

4.3 Immunohistochemistry Investigations

4.3.1 Using TSA Enhancing Technique in Detecting HIV-1 p24 Antigen in Various Organs

At least two serial sections were taken from each tissue block following the standard procedure as described in section 2.5. The screening detection of p24 antigen was carried out following the TSA technique with optimised conditions. To avoid mis-reading a false positive pattern, one of the two serial sections was not exposed to the anti-HIV-1 p24 antibody, and this served as a negative reference for the sequential section. Additional positive control using a block of blown HIV-1 p24 positive tissue, and negative control that using normal brain cortex tissue from the non-HIV-positive individual were required for each run of experimental staining.

4.3.2 Analysis of Cellular Localisation of HIV-1 Infection

Tissue blocks that showed p24 immunopositivity were selected for investigating the cellular localisation by double-labeling IHC or serial staining with anti-p24 antibody and various cell markers. At least 6 serial sections were taken from each block and staining for various cell markers was undertaken as described in Table 4. 1.

Generally, antibodies for T cells (CD3 and CD8), macrophages and brain microglia (PG-M1), B cells (L26), and follicle dendritic cells (CD21) were used. Any p24 positive cells that were not recognised by the cell markers listed above were stained using other cell markers, such as GFAP (for astrocyte), depending on the observed morphology.

Table 4. 1. Working plan of immunocytochemistry on serial sections for the investigation of HIV-1 cellular localisation.

No.	Antibody	Target
1	L26 (CD20)	Reacts with the majority of B cells
2	CD21	A restricted B cell antigen expressed on mature B cells. This antibody shows strong labelling of FDC in paraffin sections.
3	P24	HIV-1 p24 antigen
4	PG-M1 (CD68)	Macrophage lineage, including Brain microglial cells
5	CD3	Majority of T cells
6	CD8	Cytotoxic/suppressor CD8 T cell subset

* Details of working conditions for each antibody were listed in section 2.5

4.3.3 Investigations of *in vivo* Distribution of CXCR4 and CCR5 Receptors

Staining with co-receptor antibodies proved unpredictable unless fixation time was short. Therefore short-fixed samples from nine recent cases were used for investigating the general distribution of CXCR4 and CCR5 co-receptors. At least three sections were taken from each tissue block, and stained with CXCR4, CCR5 and normal blocking serum (as a negative control) respectively using the ABC technique.

4.4 Results and Discussion

Evidence of HIV-1 replication in each tissue was demonstrated by immunocytochemical staining for p24 antigen. The application of the TSA technique gave a great enhancement in p24 antigen detection in various organs. Especially in formalin-fixed, paraffin-embedded non-lymphoid tissues, such as lung, liver and kidney, which were previously p24 negative, weak but positive signals were detected while using the TSA technique.

Key for Table 4. 2**:**

[a]: Risk Groups:

PDM --- pre-AIDS Drug Misuser;	**ADM** --- AIDS Drug Misuser
PH --- pre-AIDS Homosexual;	**AH** --- AIDS Homosexual
P-Hetero --- pre-AIDS heterosexual;	**A-Hetero** --- AIDS heterosexual
A-Btrans --- AIDS blood transmission;	**A-Haem** --- AIDS haemophilia

[b]: Drug Therapy:

0 --- None

1 --- Less than 6 weeks AZT in total

2 --- More than 6 weeks but not in last year of life

3 --- More than 6 weeks within one year of death

N/A --- not available

[c]: Cognitive Impairment: Cognitive function has been described using a range of neuropsychological measures, including an estimate of premorbid IQ, measures of performance speed, current intellectual function, memory and mood. A neurophysiological test, the auditory event-related potential test, was also used to determine the onset of a subcortical dementing process. This test offers a non-specific physiological assessment of cognitive function, sensitive to the effect of subtle early brain disease, which has been shown to be abnormal in latency and amplitude in individuals with cortical and subcortical dementias. Depending on the results of sequential tests, patients were assessed as having normal cognitive function or as having mild, moderate or severe impairment if the auditory event-related potential was delayed at least 2 SD from the mean (summarised from Bell *et al.,* 1998).

[d]: Tissues:

Bv --- Blood vessel	**LU** --- Lung
CO --- Colon	**MU** --- muscle
Ile --- Ileum	**PA** --- Pancreas
KI --- Kidney	**SA** --- Salivary Gland
LF --- Left frontal lobe of brain	**SP** --- Spleen
LI --- Liver	**TY** --- Thymus
LN --- Lymph Node	

[e]: IHC results:

0: p24 negative;	**1**: p24 weakly positive
2: p24 intermediately positive;	**3**: p24 strongly positive

[f]: The number of tissue blocks examined from each organ is indicated in brackets.

Shaded area: pre-AIDS patients

Table 4. 2. The results of p24 TSA detection in a variety of organs in 53 HIV-1-infected individuals. The order depends on last CD4 counts (descending).

PatientNo/ Risk Group[a]	Age/Gender	Last CD4 Counts	Cognitive Impairment[b]	Drug Therapy[c]	Tissues examined[d]
94-132 PDM	44/M	N/A	None	0	SP: 0[e] (x2)[f] LN: 1 (x1) LF: 0 (x1) MU: 0 (x1)
97-097 PDM	36/M	N/A	None	N/A	SP: 2 (x1) LN: 3 (x1) LU: 0 (x2) LF: 1 (x1) TY: 2 (x1)
94-034 PDM	41/M	1050	None	0	SP: 3 (x1) LN: 2 (x1) LF: 0 (x1)
98-028 PDM	48/F	703	None	0	SP: 0 (x1) LN: 3 (x4); 1 (x1) LU: 1 (x1); 0 (x1) CO: 0 (x4) LF: 0 (x1) TY: 0 (x1)
95-318 PDM	40/M	496	None	0	SP: 3 (x1)
93-094 PDM	31/M	425	None	0	SP: 1 (x1)
98-025 P-Hetero	43/M	297	None	0	SP: 3 (x1); 1 (x1) LN: 3 (x8); 1 (x1); 0 (x1) LU: 1 (x1); 0 (x1) CO: 2 (x2); 0 (x5) LF: 0 (x1)
95-040 P-Hetero	31/F	247	None	0	SP: 1 (x1) LN: 1 (x1) LU: 0 (x1) CO: 0 (x1) LF: 0 (x1) MU: 0 (x2)
95-270 ADM	23/F	246	None	2	SP: 1 (x1) LN: 3 (x3) LU: 2 (x2) SA: 3 (x1)
95-066 PDM	39/F	245	None	0	SP: 1 (x1) LN: 1 (x2) LU: 1 (x1) CO: 0 (x1)
93-357 PDM	28/F	200	None	1	SP: 0 (x1) LN: 0 (x3) LF: 0 (x1)
94-171 ADM	29/F	150	None	0	LN: 1 (x1); 0 (x1) LF: 0 (x1)

Table 4. 2. The results of p24 TSA detection in a variety of organs in 53 HIV-1-infected individuals. The order depends on last CD4 counts (descending). (continued)

PatientNo/ Risk Group	Age/Gender	Last CD4 Counts	Cognitive Impairment	Drug Therapy	Tissues examined
97-021 ADM	49/F	137	Severe	2	SP: 3 (x1) LN: 3 (x1) LU: 2 (x1); 0 (x1) CO: 0 (x2) LF: 3 (x1)
96-446 ADM	31/F	136	Mild	1	SP: 3 (x2) LN: 3 (x2) TY: 1 (x1)
96/272 ADM	32/M	126	None	0	SP: 3 (x2) LN: 1 (x1) LU: 0 (x2) CO: 2 (x1) Ile: 0 (x1) LF: 0 (x1)
93-358 ADM	28/M	90	Severe	2	CO: 0 (x1) LF: 3 (x1)
92-118 ADM	34/M	80	Severe	1	SP: 2 (x1) LN: 3 (x2); 1 (x1) LU: 2 (x1) Ile: 3 (x1) CO: 0 (x1) KI: 1 (x1) LF: 1 (x1)
94-162 ADM	34/M	80	None	0	SP: 3 (x1) LN: 3 (x3)
95-363 AH	40/M	69	None	0	SP: 3 (x2) LN: 0 (x2) LI: 1 (x1) TY: 1 (x1) Mubv: 1 (x1)
92-345 A-Hetero	27/F	67	None	1	SP: 1 (x2) LN: 1 (x1); 0 (x1) LU: 0 (x4) CO: 0 (x2) LI: 0 (x1) PA: 0 (x1)
96-160 AH	45/M	41	None	0	SP: 2 (x1); 0 (x1) LN: 3 (x2) LU: 0 (x2)
94-271 ADM	35/F	39	None	0	SP: 0 (x1) LN: 1 (x2) LF: 0 (x1) TY: 0 (x1)
95-199 ADM	32/F	28	Mild	2	SP: 1 (x2) LN: 1 (x3) LU: 1 (x2); 0 (x3) CO: 0 (x1)

Table 4. 2. The results of p24 TSA detection in a variety of organs in 53 HIV-1-infected individuals. The order depends on last CD4 counts (descending). (continued)

PatientNo/ Risk Group	Age/Gender	Last CD4 Counts	Cognitive Impairment	Drug Therapy	Tissues examined
95-310 AH	47/M	27	None	NA	SP: 1 (x1) LN:0 (x3) LU: 0 (x1) TY: 0 (x1)
94-286 ADM	45/M	23	None	0	SP: 0 (x1) LI: 0 (x1) LF: 0 (x1)
96-267 ADM	36/F	23	None	2	SP: 0 (x2) LN: 0 (x6) LU: 0 (x2) LI: 0 (x3) KI: 0 (x1) AD: 0 (x1) MU: 0 (x2)
93-087 AH	38/M	22	None	2	SP: 1 (x1) LU: 1 (x1); 0 (x1) Ile: 0 (x2) CO: 0 (x1) LI: 0 (x2) KI: 1 (x2) TY: 0 (x1)
95-108 A-Btrans	52/F	22	Severe	3	SP: 3 (x1) LN: 1 (x2); 2 (x1) LU: 1 (x1) CO: 0 (x1) LF: 1 (x1) TY: 0 (x1)
94-186 ADM	41/M	19	Mild	2	SP: 0 (x1) LF: 0 (x1)
96-013 ADM	35/M	18	None	0	SP: 0 (x2) LN: 0 (x2) LU: 0 (x2) CO: 0 (x1) LI: 0 (x1) KI: 0 (x2) TY: 0 (x1)
97-017 ADM	32/M	16	None	3	SP: 0 (x1) LN: 0 (x1) LU: 0 (x3) CO: 0 (x3) LF: 0 (x1)
91-420 ADM	27/F	12	None	1	SP: 1 (x1) LN: 0 (x1) LU: 0 (x3) Ile: 0 (x1) CO: 0 (x2) LI: 0 (x1) KI: 0 (x1) LF: 1 (x1)

Table 4. 2. The results of p24 TSA detection in a variety of organs in 53 HIV-1-infected individuals. The order depends on last CD4 counts (descending). (continued)

PatientNo/ Risk Group	Age/Gender	Last CD4 Counts	Cognitive Impairment	Drug Therapy	Tissues examined
94-001 A-Hetero	34/F	9	None	1	SP: 0 (x1) LN: 0 (x2) Ile: 0 (x2) CO: 0 (x1)
94-170 ADM	39/F	9	Mild	2	SP: 2 (x2) LF: 0 (x1)
95-060 ADM	43/F	9	Moderate	2	LU: 0 (x2) LI: 0 (x1)
96-206 AH	37/M	8	Mild	2	SP: 1 (x1) LN: 3 (x2) TY: 1 (x1)
97-020 AH	49/M	8	Severe	1	SP: 0 (x2) LN: 0 (x1) LU: 0 (x3) CO: 0 (x2) Ile: 0 (x2) LF: 2 (x1)
92-218 AH	30/M	4	Moderate	0	SP: 2 (x1) LN: 1 (x2); 0 (x1) LU: 0 (x3) Ile: 0 (x2) LI: 0 (x1) KI: 0 (x3)
94-038 ADM	29/M	3	Mild	2	SP: 3 (x1) LN: 3 (x1); 1 (x1) LF: 3 (x1)
96-279 AH	39/M	3	Moderate	2	SP: 3 (x2) LN: 2 (x2) LU: 0 (x1) Ile: 0 (x1) TY: 1 (x1); 0 (x1)
96-371 AH	34/M	3	Mild	3	SP: 3 (x1) LN: 1 (x1) LU: 0 (x2) Ile: 0 (x1) CO: 0 (x2) LF: 0 (x1)
91-246 ADM	32/M	2	Mild	2	SP: 1 (x1) LU: 3 (x1) ; 0 (x3) CO: 1 (x1) LF: 0 (x1) MU: 0 (x1)

Table 4. 2. The results of p24 TSA detection in a variety of organs in 53 HIV-1-infected individuals. The order depends on last CD4 counts (descending). (continuing)

PatientNo/ Risk Group	Age/Gender	Last CD4 Counts	Cognitive Impairment	Drug Therapy	Tissues examined
95-079 A-Hetero	35/F	2	Severe	2	LN: 0 (x1) LU: 0 (x3) CO: 0 (x1) LF: 1 (x1) TY: 0 (x1)
96-425 ADM	32/M	1	Mild	0	SP: 3 (x1) LN: 1 (x1) LU: 1 (x1); 0 (x2) CO: 0 (x1) Ile: 0 (x2) LF: 0 (x1) TY: 0 (x1)
94-276 A-HAEM	20/M	0	None	1	LN: 0 (x1) LF: 0 (x1)
94-313 AH	41/M	0	None	0	LN: 0 (x2) LF: 0 (x2) PA: 0 (x1)
95-088 A-Hetero	22/M	0	None	N/A	SP: 1 (x1); 0 (x1) LN: 1 (x3); 0 (x1) LU: 1 (x1); 0 (x2) LF: 1 (x1) TY: 0 (x1)
95-295 AH	34/F	0	None	0	SP: 1 (x1) LN: 1 (x1) LU: 0 (x1)
95-371 AH	49/M	0	Mild	0	SP: 1 (x1) LN: 1 (x2); 0 (x1) TY: 0 (x1)
95-388 AH	29/M	0	Mild	3	SP: 1 (x1) LN: 1 (x1) LU: 0 (x1) LI: 0 (x1) TY: 1 (x1); 0 (x2)
96-033 AH	27/M	0	Mild	N/A	SP: 0 (x1) LN: 0 (x2) LU: 0 (x1) Ile: 0 (x1) CO: 0 (x2) LI: 0 (x1) KI: 0 (x1) SA: 0 (x2)
96-162 A-Hetero	52/F	0	Severe	2	SP: 0 (x1) LN: 0 (x1)
96-173 ADM	34/M	0	Mild	2	SP: 0 (x3) LN: 0 (x2) LF: 1 (x1) TY: 0 (x2) SA: 0 (x1)

This immunohistochemistry study demonstrated a close association between p24 detection in brain tissue and advanced disease, although this kind of association was not observed within LN and spleen, which were positive at all stages of infection. In summary, during the clinically latent stage, p24 signal was often detected within lymphoid organs and occasionally in lung and colon sections; once advanced to the symptomatic stage, p24 signal was detected within a wider range of organs, including LN, spleen, lung, intestine, liver, kidney and brain (Table 4. 2 & Table 4. 3).

Table 4. 3. Summary of the HIV-1 p24 antigen detection in various organs

	Pre-AIDS	**AIDS**
	No. of positive /No. of tested (%)*	No. of positive /No. of tested (%)*
Spleen	8/12 (67%)	33/51 (65%)
Lymph Node	20/24 (83%)	43/75 (57%)
Lung	3/8 (38%)	14/59 (24%)
Ileum	N/A	1/16 (6%)
Colon	2/13 (15%)	2/25 (8%)
Left Frontal Block	1/7 (14%)	10/23 (43%)
Liver	0/2 (0)	1/13 (8%)
Kidney	N/A	2/11 (18%)
Thymus	1/2 (50%)	4/19 (26%)
Salivary Gland	N/A	1/4 (25%)
Muscle + Bv**	0/3 (0)	1/4 (25%)
Pancreas	0/1 (0)	0/2 (0)

* Number was counted according to the tissue blocks, not by patients

** Bv: blood vessel

N/A: Not Available

4.4.1 Cellular Localisation and General Distribution of HIV-1 p24 Antigen in the Lymphoid Organs

Lymph Node and Spleen

In this study, immunohistology revealed the presence of p24 positive signal in LN and spleen sections throughout the course of infection, even in recently deceased individuals who had received effective trial suppression therapy (Table 4. 2, Table 4. 3 & Table 4. 4). The staining pattern of HIV-1 p24 was characterized by strong cytoplasmic granular staining. The majority of p24 immunopositive cells in spleen and LN sections were observed primarily in germinal centres of lymphoid follicles. Only in a few instances, p24 positive cells were present in the paracortical area.

The level of p24 staining intensity varied between and within individuals (Figure 4. 1), however, the difference neither correlated with risk group nor with AIDS-related illness (Table 4. 2 & Table 4. 4). For example, varying intensity and positivity of p24 staining was detected and distributed evenly in the spleen and LN sections taken from pre-symptomatic and symptomatic in the drug misuser risk group. In each risk group, there existed patients who were p24 negative in lymphoid tissue, and also, there were sections that stained p24 strongly positive. One interesting finding is that the intensity of p24 staining was slightly correlated with last CD4+ cell counts (Spearman rank correlation coefficient 0.8 (LN) & 0.4 (spleen); Table 4. 5). Most sections from patients with higher CD4+ cell counts present a higher level of p24 positivity, and sections from those whose last CD4+ cell counts were zero were usually p24 negative. In general, relatively normal histology, or follicular hyperplasia, was observed in these spleen and LN sections taken from individuals with higher CD4+ cell counts, usually from pre-symptomatic individuals. When patients advanced to the symptomatic stage, the CD4+ cell counts decreased, and most cases were reported to show atrophy and severe lymphocyte depletion in LNs and spleen. Because the HIV-1 p24 positive signal was localised in the germinal centres of follicles, the destruction of follicles therefore resulted in reduced p24 positivity within sections taken from symptomatic

individuals, and this could explain the correlation observed between last CD4+ counts and the intensity of p24 immunopositivity. However, not all germinal centres in a section were equally affected. Generally, in a section, the level of the germinal centre destruction, and the intensity of p24 staining varied from follicle to follicle (Figure 4. 1).

Table 4. 4. Comparison of the p24 staining positivity and the risk group in spleen and lymph node sections from different stages of disease.

	Spleen		Lymph node	
P24 Score	**Pre-AIDS** (total 12)	**AIDS** (total 51)	**Pre-AIDS** (total 24)	**AIDS** (total 75)
	No. of positive	No. of positive	No. of positive	No. of positive
0	PDM: 4 (33%)*	ADM: 11 (22%)** AH: 4 (8%) A-Hetero: 3 (6%)	PDM: 3 (25%)* P-Hetero: 1 (8%)	ADM: 13 (17%)** AH: 12 (16%) A-Hetero: 6 (8%) A-Haem: 1 (1%)
1	PDM: 2 (17%) P-Hetero: 2 (17%)	ADM: 5 (10%) AH: 6 (12%) A-Hetero: 3 (6%)	PDM: 4 (33%) PH: 1 (8%) P-Hetero: 2 (17%)	ADM: 10 (13%) AH: 7 (9%) A-Hetero: 4 (5%) A-Btrans: 2 (3%)
2	PDM: 1 (8%)	ADM: 2 (4%) AH: 3 (6%)	PDM: 1 (8%)	AH: 2 (3%) A-Btrans: 1 (1%)
3	PDM: 2 (17%) P-Hetero 1 (8%)	ADM: 8(16%) AH: 5 (10%) A-Btrans: 1 (2%)	PDM: 5 (42%) P-Hetero: 8 (67%)	ADM: 12 (16%) AH: 4 (5%)

* positive cases / total examined cases of spleen/LN from pre-AIDS individuals
** positive cases / total examined cases of spleen/LN from AIDS individuals

Table 4. 5. The correlation between p24 immunopositivity and the CD4 counts at death in LN and Spleen.

	Score 0	Score 1	Score 2	Score 3	**Spearman rank correlation coefficient**
CD4 counts* in Spleen	57.4	95.6	28.6	126.18	0.4
CD4 counts in LN	43.53	83.52	269.5	236.23	0.8

* the average of CD4+ cell counts in each range

Figure 4. 1. The detection of HIV-1 p24 in LN and spleen. In general, p24 immunopositivity is localised in the germinal centres of lymphoid follicles. The staining pattern is characterised by strong cytoplasmic granular staining. The level of p24 staining intensity varied between and within individuals. (DAB with haematoxylin counter stain. Magnification: x25 (A-D); x50 (E); x200(F)).

(A): LN section from pre-AIDS drug misuser

(B): LN section from AIDS drug misuser with lymphocyte depletion

(C) & **(D)**: Two different areas from one spleen section taken from AIDS heterosexual individual. A high level of p24 positivity in view **(C)**, but only few cells stained (with indicators) in view **(D)**.

(E): LN section with pre-AIDS drug misuser with lymphocyte depletion

(F): A representative p24 staining pattern, usually cytoplasmic granular staining, often along with the processes (indicated with arrows) of follicular dendritic cells

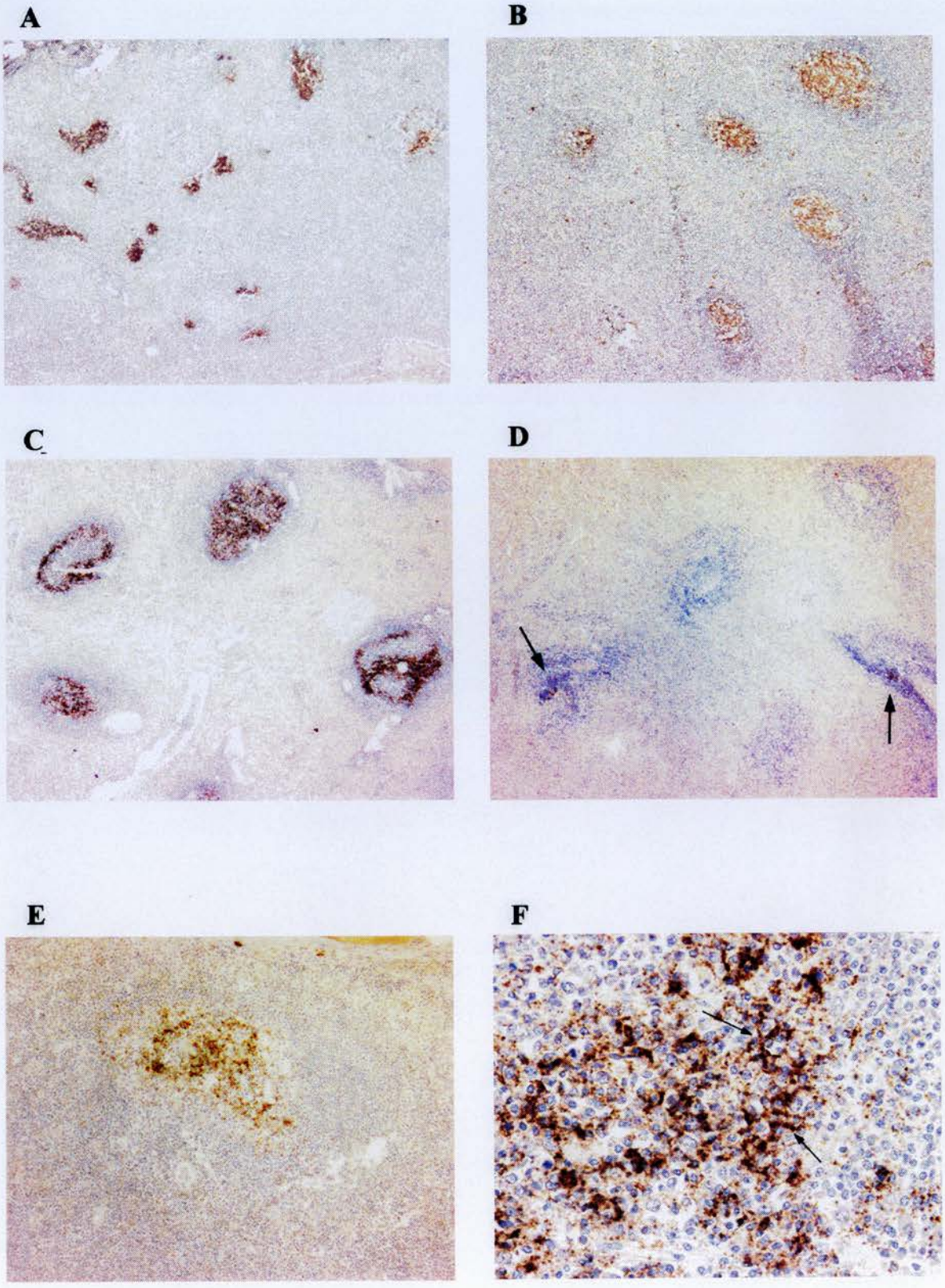
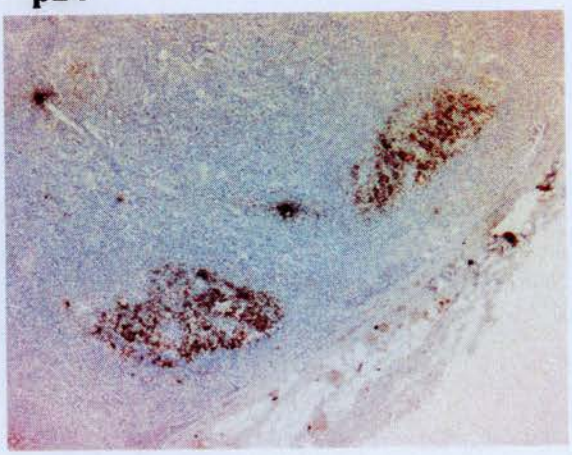


Fig. 4.1

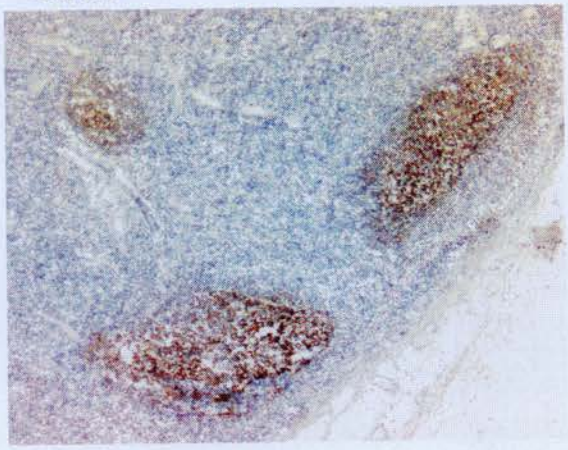
Lymphoid organs are the anatomic sites where the generation and propagation of antigen-specific immune responses occur (Pantaleo *et al.*, 1993b; Pantaleo *et al.*, 1994). During acute infection, peripheral antigen-activated T lymphocytes (predominantly CD4+ cells) or other antigen presenting cells (APC) migrate from the circulation and the lymphatics into the interface between the paracortex (T-cell zone) and the germinal center (B-cell zone) in order to provide help for the generation of antigen-specific B-cell responses against HIV (Pantaleo *et al.*, 1993b; Pantaleo *et al.*, 1994). There are various cell populations in the spleens and LNs, including B and T lymphocytes, macrophages, interdigitating cells and FDCs. Some of these resident cells, such as CD4+ T lymphocytes, macrophages, interdigitating cells are susceptible to HIV-1 infection (Embretson *et al.*, 1993a; Pantaleo *et al.*, 1994; McIlroy *et al.*, 1996; Reinhart *et al.*, 1997; Patterson *et al.*, 1998; Soontornniyomkij *et al.*, 1998). However, in this study, the viral reproductive signal was found primarily in the germinal centres of lymphoid follicles. Germinal centers contain resting B cells clustered around a dense network of processes extending from follicular dendritic cells, and a few macrophages. Most of the p24 positive signals in the germinal centres co-localised with large mononucleated or binucleated cells with elaborate cytoplasmic extensions. According to the staining results of serial sections, the pattern of p24 staining was similar to the FDC network shown by anti-CD21 (Dako) staining, which is well suited for detection of FDCs in formalin-fixed paraffin-embedded tissues (Figure 4. 2). Later using a double-immunolabelling technique, p24 and CD21 staining patterns could be evaluated simultaneously (Figure 4. 2). In contrast to the p24 staining of varying frequency which was readily shown in the germinal centres in almost all specimens, isolated p24 positive cells were rarely observed elsewhere in sections. For example, in the T-cell-dependent areas of spleen and LN sections, which stained intensely for CD3 and CD8, limited CD3-positive cells were double-stained with anti-p24 mAb, but no co-localisating signal was observed between CD8 and p24 (Figure 4. 3). Although CD4 detection was not successful in this study (refer to section 2.6.4.1), the rare observation of p24 positive cells outside the germinal centres suggested an infrequent productive infection of T lymphocytes and macrophages.

Figure 4. 2. A panel of serial sections of lymph node stained with p24, CD21, CD3, PGM1, CD8 and L26 demonstrating the staining pattern of p24 is similar to the CD21 staining pattern (DAB with haematoxylin counter stain. Magnificataion, x50).

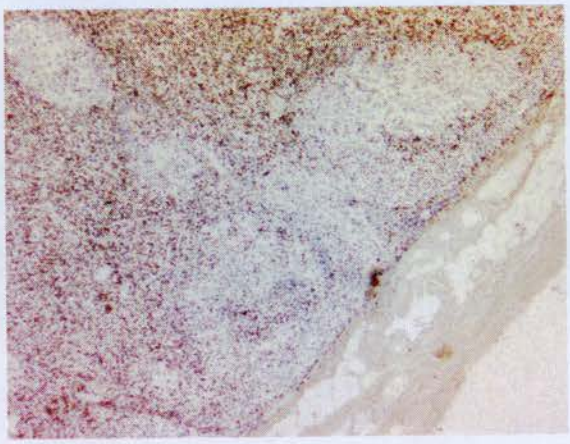
p24



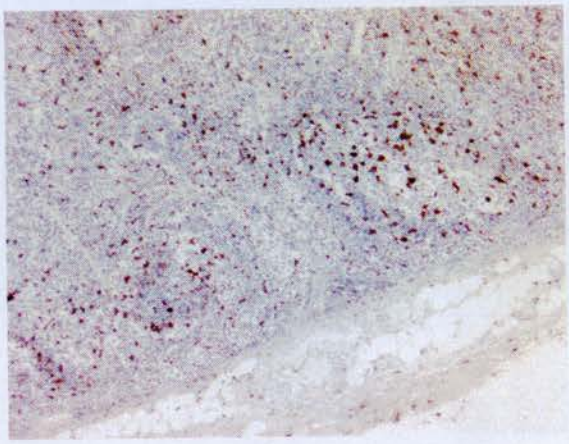
CD21



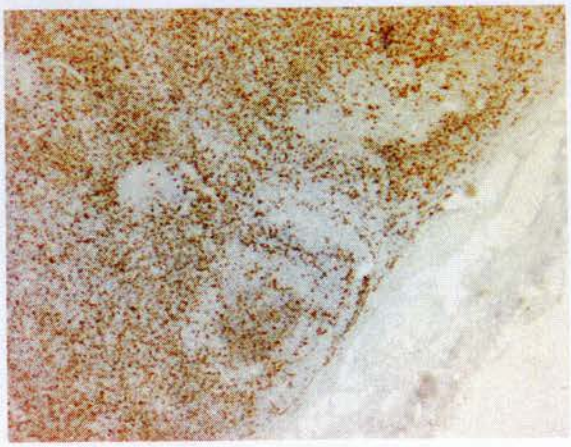
CD3



PGM1



CD8



L26

Fig. 4.2

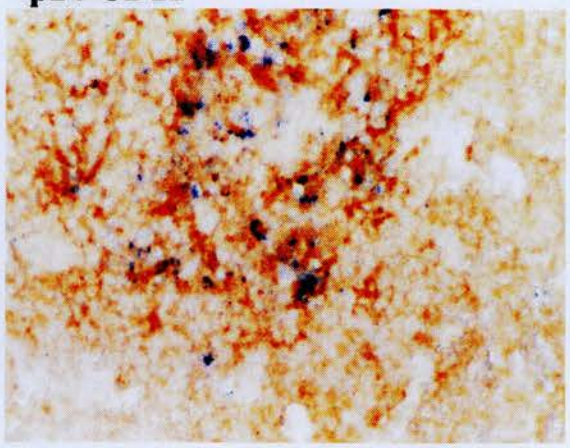
Figure 4. 3. Double immunolabelling staining for p24/CD21, p24/CD8 and p24/CD3 in LN section.

Top: p24 positivity (Vector Blue) is colocalised with CD21 positivity (DAB). (Magnificant: left: x200, right: x100).

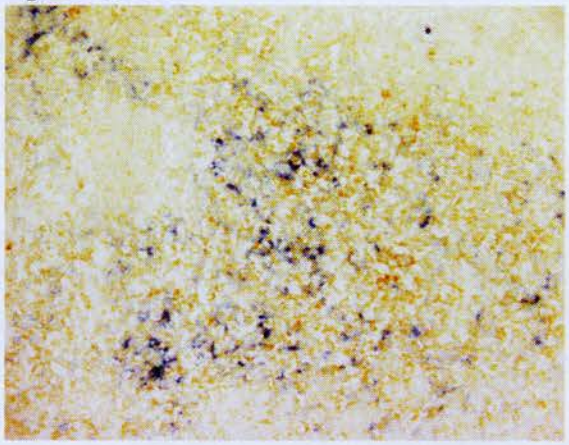
Middle: p24 positivity (DAB) is infrequently observed within CD3 positive cells (Vector Blue) (with indicators). (Magnification: left: x400; right: x200).

Bottom: No colocalisation was observed between p24 (DAB) and CD8 positive cells (Vector Blue). (Magnification: left: x400; right: x200).

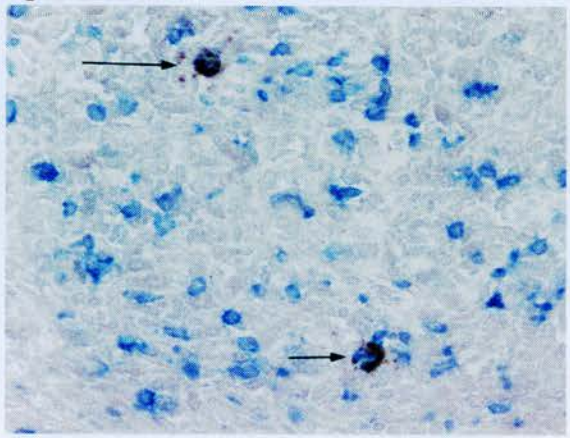
p24-CD21



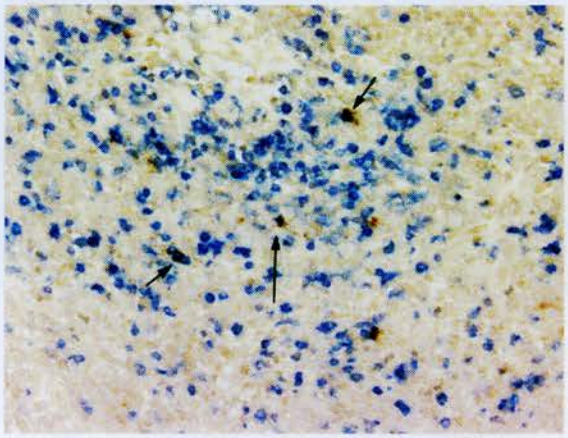
p24-CD21



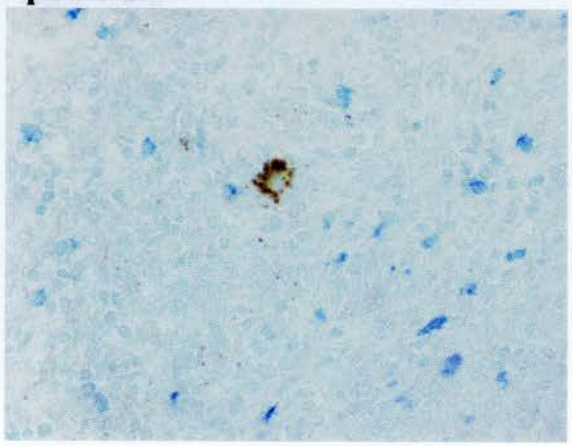
p24-CD3



p24-CD3



p24-CD8



p24-CD8

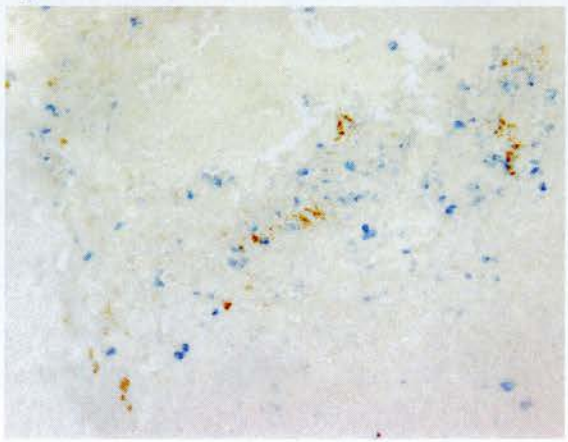


Fig. 4.3

Unlike the bone-marrow derived dendritic cells that present peptide antigens to CD4+ T lymphocytes and comprise a system that occupies discrete portions of non-lymphoid and lymphoid organs (Knight, 1996), FDCs do not express MHC class II molecules and are found only in the germinal centres of lymphoid follicles in the LN, spleen and mucosa-associated lymphoid tissues (Abbas *et al.,* 1999). Basically, FDCs express high-affinity receptors for complement proteins and for the Fc regions of IgG molecules, therefore they are able to capture intact virus particles or viral antigens in antigen-antibody complexes on their surfaces soon after seroconversion (Abbas et al., 1999). Antigens or intact virus particles can persist in this form of immune complexes for long periods (Janeway & Travers, 1996). Whether FDCs are infected *in vivo* with HIV-1 or merely have HIV-1 trapped on their surfaces as immune complexes has not been resolved. Several studies have found evidence of *in vivo* productive infection of FDCs, such as virus particles budding from FDC membrane (Armstrong & Horne, 1984; reviewed in Tenner-Racz *et al.,* 1994), HIV-1 envelope messenger RNA detected in FDCs, and HIV-1 RNA detected in FDC perinuclear cytoplasm (Spiegel *et al.,* 1992; reviewed in Levy, 1998). However, most investigators believed that HIV particles were trapped on the villus processes of FDCs as immune complexes (virus:complement-antibody complexes) and that the cells were not infected (Pantaleo *et al.,* 1993a; Embretson *et al.,* 1993a; Schmitz *et al.,* 1994; Tenner-Racz *et al.,* 1994; Tsunoda *et al.,* 1996; Orenstein *et al.,* 1997b; Reinhart *et al.,* 1997). In my study, a non-uniform immunostaining for HIV-1 p24 was observed in all LN and spleen sections and this observation is similar to that of Soontornniyomkij *et al.,* who used double immunofluorescent staining followed by confocal microscopy for HIV-1 p24 detection (Soontornniyomkij *et al.,* 1998). In general, within a section, some follicles were found to be heavily stained but adjacent follicles were negative. Soontornniyomkij *et al.* concluded that such a non-uniform staining pattern, together with the observed intimate colocalisation of p24 and CD21, gave evidence that FDCs could be infected and could express p24 in their cellular processes, and did not support the hypothesis that HIV-1 was bound to FDCs surface as extracellular antibody-coated complexes. In that regard, a rather uniform involvement of all follicles within an individual lymphoid structure would be

predicted. Nevertheless, using an immunostaining technique alone, it is difficult to state whether or not FDCs are productively infected.

The observation that high levels of p24 immunopositivity were persistently confined within the FDCs in the LN and spleen from presymptomatic and symptomatic subjects is similar to previous reports, which used different methodologies to detect productive signal in LNs from various stages of disease (Pantaleo *et al.,* 1993a; Pantaleo *et al.,* 1993b; Embretson *et al.,* 1993a; Haase *et al.,* 1996; Blauvelt *et al.,* 1997; Pantaleo *et al.,* 1998), and these FDC-entrapped virus were highly infectious (Heath *et al.,* 1995). These observations suggest that HIV infection is active and progressive in lymphoid tissues throughout the course of HIV infection (Haase *et al.,* 1996; Blauvelt *et al.,* 1997; Pantaleo *et al.,* 1998). Although it is difficult to determine whether FDCs are capable of being infected whether or not the infection is rare or inefficient, it is believed that these cells do play an essential role in HIV-1 pathogenesis. Clinically, a common finding in end-stage disease is that the lymphoid organs lose their normal architecture owing to the slow but persistent destruction of CD4+ cells and FDCs (Rosok *et al.,* 1997; Pantaleo *et al.,* 1998). A loss of FDC might reduce efficient antigen presentation and the ability of the host to generate strong antiviral immune responses with consequent risk of severe diseases (Knight, 1996). The cause of the degeneration and death of FDCs is not yet clear, which could be a cytopathic effect of HIV-1 or T-cell-mediated cytotoxicity (Gray *et al.,* 1996; Esser *et al.,* 1998). Nevertheless, it is believed that releasing virus from intercellular trapping in the FDC network or producing virus from infected cells could be a continuing source of infection (Pantaleo *et al.,* 1993a; Pantaleo *et al.,* 1993c; Pantaleo *et al.,* 1995; Haase et al., 1996; Reinhart *et al.,* 1997). Recent studies have found that FDC-entrapped virions are not affected by antiretroviral therapy (Haase *et al.,* 1996; Pantaleo *et al.,* 1998). Even using a combination regimen, the effects are limited (Montaner *et al.,* 1998b; Orenstein *et al.,* 1999b). Thus, FDCs have been though to be a reservoir for HIV-1, so that on progress to the symptomatic stage of disease, the persistent FDC-entrapped viruses released because of the disruption of the FDC network, and then transmitted to surrounding CD4+ cells, or released into the circulation leads to the

rapid HIV-1 viremia rebound observed after the interruption of HAART (Orenstein *et al.,* 1999b). In this regard, the development of new therapeutic strategies should focus more on these cells.

Thymus

Most of the thymus sections showed considerable thymic atrophy and severe lymphocyte depletion. In one section taken from a pre-symptomatic individual (NA97097), lymphofollicular hyperplasia was observed. Using the enhancing TSA technique, HIV-1 p24 antigen was detected in five of twenty-one blocks, four blocks taken from AIDS cases and one from a pre-AIDS individual. The intensity of p24 immunopositivity observed in the four sections taken from symptomatic stages was very low. P24 signal was only detected in a few scattered cells, in which p24 immunostaining overlapped CD3 positivity and morphologically the positive cells appeared to be lymphocytes. In the section taken from the pre-symptomatic stage, p24 positive cells were observed in the lymphoid follicles. The staining pattern, similar to the LN and spleen, was reticular and irregular suggesting the labeling of the follicular dendritic cells and their cytoplasmic processes. It was confirmed later in a serial staining with various cell surface markers that these p24 positive cells were found much closer to CD21 positive cells, although the CD21 staining is weak. Also, various degrees of B-lymphocyte infiltration were observed in these p24-positive sections, especially in the one with lymphofollicular hyperplasia.

As the primary site of T-lymphocyte development during ontogeny, the thymus is another important area of study in HIV-1 infection. It has been proposed that the thymus could be the earliest target tissue infected by HIV-1 (Aldrovandi *et al.,* 1993). In one study, loss of precursor T cells and destruction of lymphoid tissue structure have been observed while directly infecting the human fetal thymus with HIV-1 in SCID-hu mice (Aldrovandi *et al.,* 1993; Bonyhadi *et al.,* 1993). Their result suggested that, in addition to the gradual disruption of the lymphoid microenvironment, direct impairment of stem cells and early T lymphocyte

precursors in the thymus may also lead to the progressive decrease or dysfunction in CD4+ T lymphocytes (Aldrovandi *et al.*, 1993; Bonyhadi *et al.*, 1993; Calabrò *et al.*, 1995; reviewed in Gaulton *et al.*, 1997). Most infected cells detected in the thymus were of the early precursor type (Valentin *et al.*, 1994; Gaulton *et al.*, 1997). Several *in vitro* studies disclosed that intrathymic T cell precursors are readily infected by HIV-1, and that double positive (CD4+CD8+) cortical thymocytes, and the major thymic cell subsets are permissive to HIV-1 infection and replication (Valentin *et al.*, 1994; Calabrò *et al.*, 1995; Gaulton *et al.*, 1997). Direct evidence of *in vivo* thymic infection was rarely documented, despite the thymic involution observed in autopsy specimens from AIDS individuals, especially in studies of paediatric patients (reivewed in Gaulton *et al.*, 1997).

It is known that the thymus is essential for formation of the T-lymphocyte repertoire during ontogeny. In contrast to fetal life, healthy children, adolescents and adults rely primarily on peripheral, thymic-independent pathways to replenish the daily turnover of mature T cells (Gaulton *et al.*, 1997). Children and adolescents may go back to regenerate a thymic-dependent functional immune repertoire under conditions of extreme immune cell depletion, but adults display a reduced capacity for T-cell regeneration overall (Gaulton *et al.*, 1997). There is no evidence that adults can reactivate thymic-dependent pathways of T-cell maturation even under extreme demand. However, the few instances in this study presenting p24 immunopositivity in the thymus gave direct evidence of thymic HIV-1 infection and suggested that follicular dendritic cells were also involved in HIV-1 infection in the thymus. Strong p24 positivity detected in a pre-symptomatic individual suggests that viral entry to thymus could occur at the early stage in HIV-1 infection. Whether thymic infection could lead to more rapid disease progression or serve only as a virus reservoir, needs further investigation.

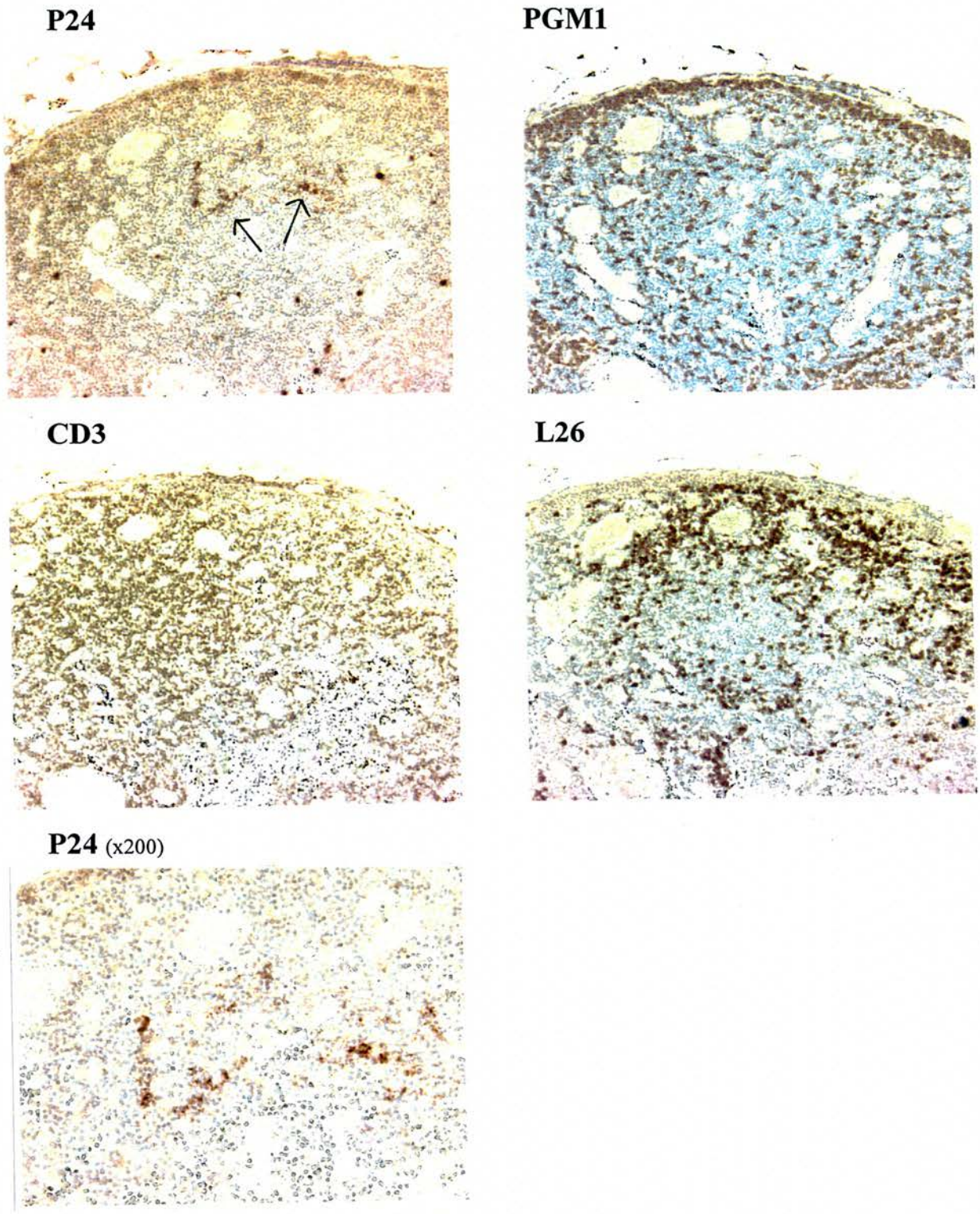


Figure 4. 4. Serial staining (p24, PGM1, CD3, L26) in thymus sections taken from a presymptomatic subject (DAB with haematoxylin counter stain. Magnification: x100). The staining pattern of p24 mAb is granular and cytoplasmic, which is similar to CD21 staining pattern (data not shown).

4.4.2 Cellular Localisation and General Distribution of HIV-1 p24 Antigen in the Brain Tissue

The detection of HIV-1 p24 immunopositivity in the brain is strongly correlated with the stage of disease. HIV-1 p24 was detected in 11 study subjects, of whom one was pre-symptomatic and ten were symptomatic patients (Table 4. 2). The ten sections taken from symptomatic subjects showing p24 immunopositivity were defined as having HIV-encephalitis (HIVE). Seven of the ten symptomatic individuals who showed p24 positivity in the brain had severe cognitive impairment. HIVE has been confirmed as a major HIV-related neuropathology in AIDS brains in the Edinburgh cohort (An *et al.,* 1996; Bell *et al.,* 1996a; Price *et al.,* 1996; reviewed in Bell, 1998), and is usually found in patients with severe AIDS related dementia (ARD). Besides severe immunosuppression and other clinical syndromes, many untreated HIV-1-infected individuals develop ARD, which may involve not only cognitive dysfunction, but also motor difficulties, coordination abnormalities and other neurological signs and symptoms (Bagasra *et al.,* 1996; Price *et al.,* 1996; reviewed in Bell, 1998). A close association between HIV-1 productive infection and HIVE has been established, although less so with ARD. However, details of molecular mechanisms involved in these HIV-1-induced dysfunctions of the CNS remain unclear.

The major HIV-1 infected cell population in the brain is the monocyte/macrophage lineage with little or no lymphocyte involvement in AIDS. This observation is different from those in the other organs, in which the p24 positive cells are usually associated with lymphoid follicles. In the brain frontal lobe sections, p24 was detected primarily in mutinucleated giant cells (MGCs), perivascular mononuclear macrophages and microglial cells, all of which could be double-stained with PGM1 (anti-CD68 mAb), a macrophage marker. The staining is granular and cytoplasmic, and is present not only in cell bodies but also in the processes of microglial cells (Figure 4. 5).

Figure 4. 5. General p24 staining patterns (DAB with haematoxylin counter stain) **and double-labelling with microglial cells** (DAB for p24 and Vector Red for PGM1 with haematoxylin counter stain) **in the brain tissue in low magnification (x25, A to D) and in high magnification (x200, E & F; x400, G to I).**

- General p24 staining pattern in grey matter **A** & **E** and in white matter **B** & **F**.
- P24 (brown) double-stained with PGM1 (red) in grey matter **C** and in white matter **D**. Most of the p24 positive cells were double-stained with PGM1, but some microglial cells remained p24 negative (as indicated with arrows).
- MGC was observed in **G** & **I** (indicated with arrow).
- Typical p24 staining in microglial cell **H**.

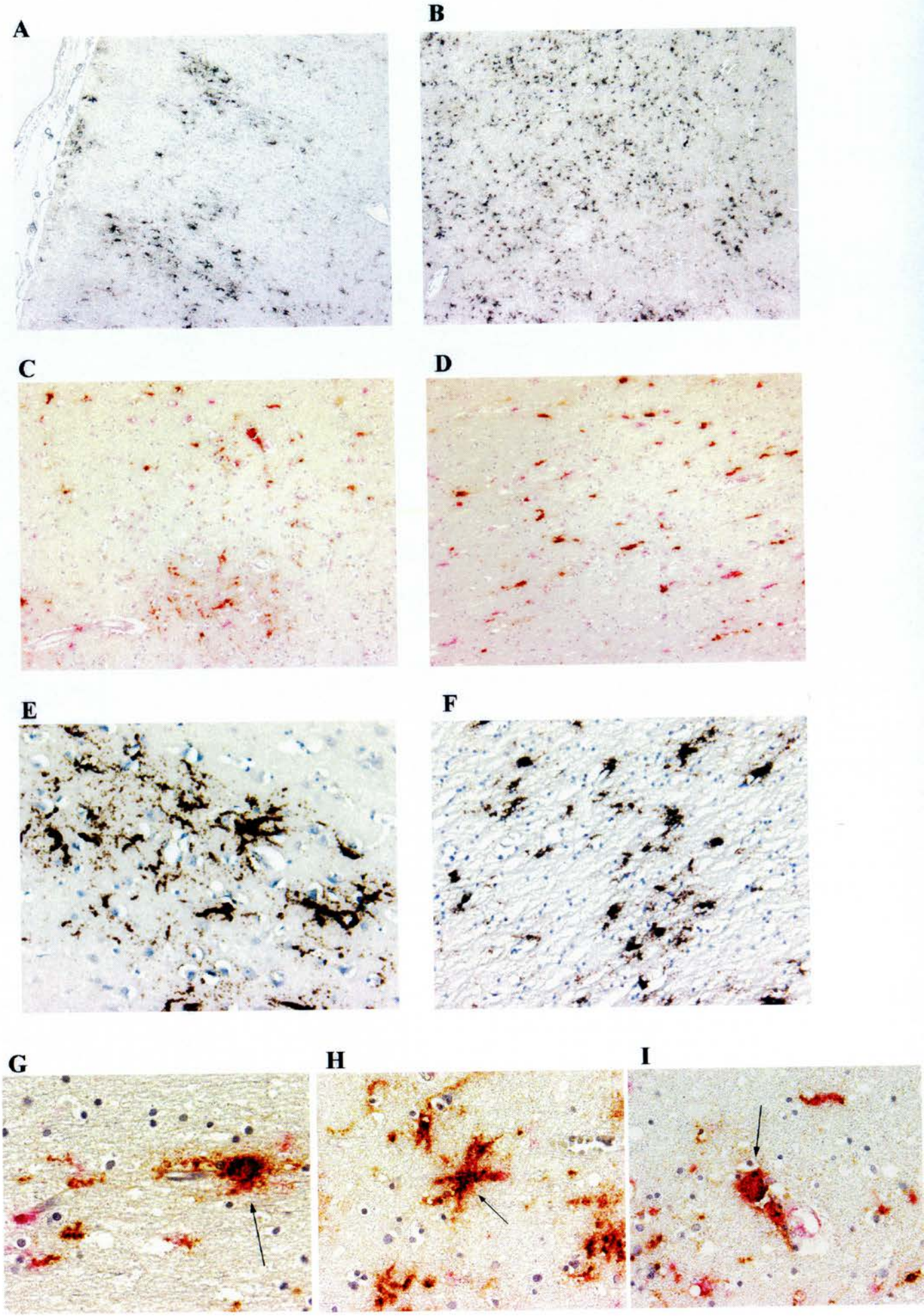


Fig. 4.5

Most of the HIV-1-infected microglial cells, macrophages, and some of the MGCs are concentrated in perivascular areas (Figure 4. 5). These findings, similar to those in previous studies (Gartner *et al.,* 1986; Wiley *et al.,* 1986; Sinclair *et al.,* 1994; Bagasra *et al.,* 1996; Gray *et al.,* 1996; Ghorpade *et al.,* 1998; reviewed in Bell, 1998), suggest that microglial cells are the major cell type to harbor the virus in the CNS.

Microglia comprise between 5 and 20 % of the total glial cell population, and form a network of rather uniformly shaped and sized cells in which each cell occupies its' defined territory (Graeber & Streit, 1990). Due to the great mutability of microglial morphology and immunophenotype, their nature as intrinsic CNS macrophage precursors has long been controversial (Graeber & Streit, 1990). Infection with HIV-1 in these cells causes a productive and cytopathic effect, and may lead to the release of several viral proteins, cytotoxic cytokines or other neurotoxic moieties from infected microglia and brain macrophages resulting in neuropathological abnormalities. Some investigators hypothesised that these neurotoxins or virotoxins, which released from infected cells will reactivate glial cells to release a number of soluble factors that are either toxic to neurons or cause chemotaxis of monocytes into brain (Nath, 1999). As the glial cells outnumber the neurons by 10 in 1, this is an important mechanism by which the virotoxins amplify their toxic potential and initiate a self-perpetuating cascade of events, resulting in a "domino effect" on the brain (Nath, 1999). However, the exact mechanisms need further investigation.

Cells of neuroectodermal origin, such as oligodendrocytes, astrocytes and neurons have been found could probably be infected with HIV-1 (Li *et al.,* 1990; Esiri *et al.,* 1991; Zauli *et al.,* 1993; Nuovo *et al.,* 1994; Tornatore *et al.,* 1994; Bagasra *et al.,* 1996; Strappe *et al.,* 1998; Brack-Werner, 1999; Hao & Lyman, 1999). No evidence of p24 positivity was observed within cells of neuroectodermal origin in this study except the observation in one case, patient 86-130 that HIV-1 p24 positive cells were double-stained with GFAP, a cell marker for astrocytes.

Figure 4. 6. Special event of p24 positivity within Astrocytes in brain tissue.

(Vector Blue for p24, and DAB for astrocytes without counter stain. Magnification: x50 in A & B, x200 in C to H, and x400 in I & J).

- A portion of astrocytes in these sections was double-stained with HIV-1 p24 mAb (panel **C** to **E**), although some of them remained p24 negative (as indicated with arrows in **J**).
- Most of the p24 immunopositivity was not colocalised with astrocytes (as indicated with arrows in panel **F** to **H**), but morphologically to be microglia.

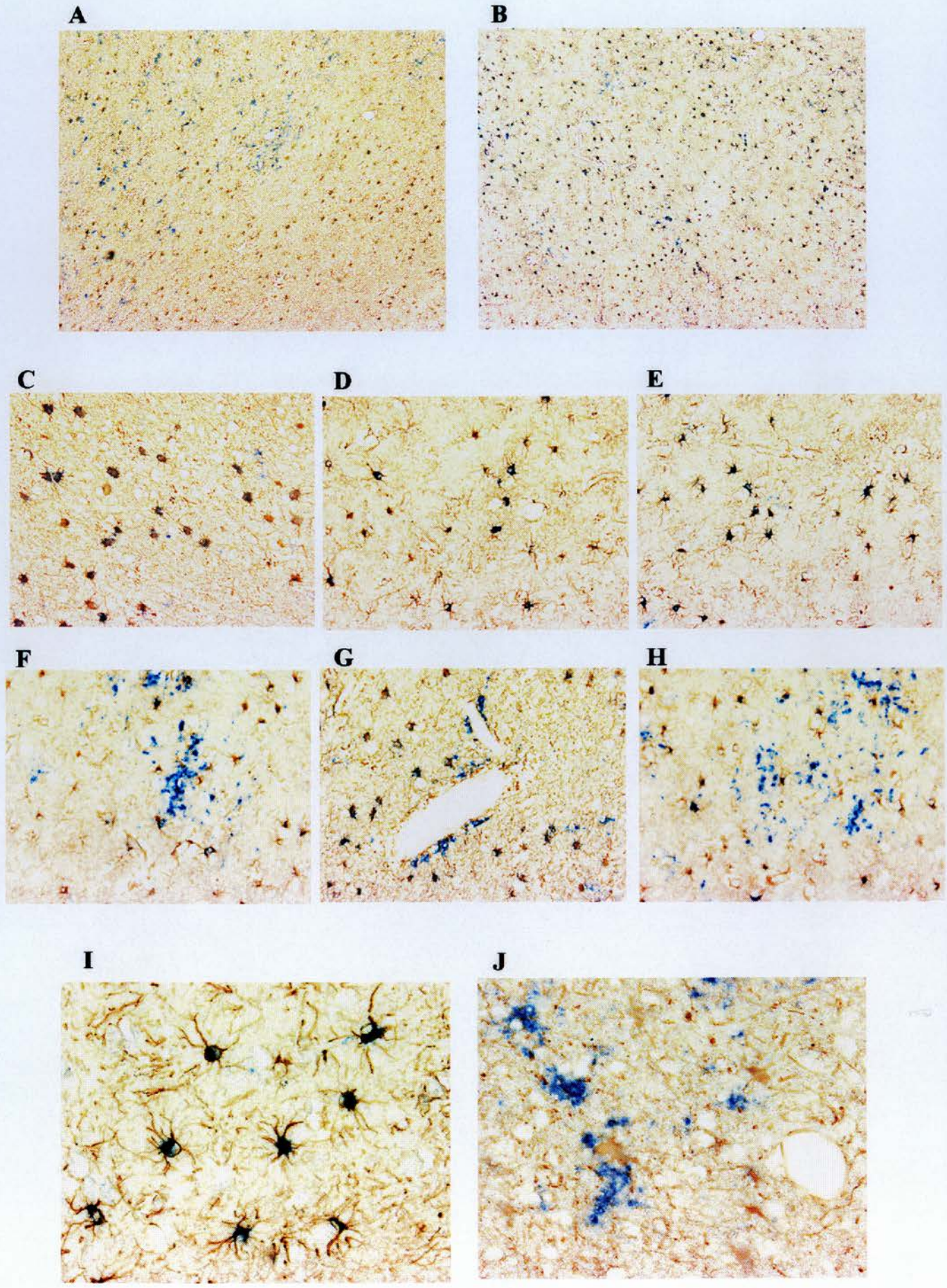


Fig. 4.6

Astrocytes are the most abundant cell type in the CNS forming a complex network which provides essential metabolites for neurons, and function to regulate K^+ ion and neurotransmitter concentrations in the neuronal extracellular milieu, and which maintains physiological barriers between the brain parenchyma and the blood or cerebrospinal fluid (CSF) (Brack-Werner, 1999). In general, direct CNS infection with HIV results in a reactive astrocytosis (Brack-Werner, 1999). Much work has been done to investigate the complex role the astrocyte seems to play in HIV infection. As we know, microglial and multinucleated giant cells are productively infected with virus during HIVE, and these infected cells produce high levels of cytokines (Nottet *et al.,* 1995) such as Tissue Necrosis Factor-α (TNF-α) and Platelet Activating Factor (PAF) both thought to be neurotoxic (Gelbard *et al.,* 1994). It has been postulated that released cytokines or viral protein (gp120) leads to the activation of surrounding astrocytes resulting in reactive astrogliosis (Epstein & Gendelman, 1993; Benos *et al.,* 1994), and this would cause the outflow of K+ and glutamate from the astrocyte leading to neuronal damage and loss which has been demonstrated in AIDS brains (Brack-Werner, 1999). Some studies have demonstrated that astroctyes could be infected occasionally by HIV-1 (Nuovo *et al.,* 1994; Saito *et al.,* 1994; Tornatore *et al.,* 1994; Brack-Werner, 1999; Nath, 1999), but the infection is silent (Ranki *et al.,* 1995; Bagasra *et al.,* 1996; Nath, 1999). Previous work in paediatric AIDS brains has demonstrated that some astrocytes are persistently infected with HIV-1 such that HIV-1 early gene products, tat, rev and nef are produced but structural viral proteins are not (Saito *et al.,* 1994). However such findings are not observed in the adult CNS. In this regard, and with the unexpected result found in this study, the infection status of astrocytes should be re-examined more carefully.

Productive HIV-1 replication in the CNS is believed to occur only at a very low level in the asymptomatic stage, although by using the PCR technique, HIV-1 proviral DNA was occasionally detected in brain tissue in asymptomatic patients (Sinclair *et al.,* 1992; Sinclair *et al.,* 1994; An *et al.,* 1996). Similar to the previous studies (Gray *et al.,* 1992; Bell *et al.,* 1993; reviewed in Bell, 1998), the only salient neuropathology present in the pre-AIDS subjects was a significant T-lymphocytic infiltration, most often CD8+ cells, in the meningeal and perivascular areas of brain tissues. The raised lymphocyte numbers in the pre-symptomatic HIV-1 positive patients might represent an immune response to early invasion of the CNS by HIV-1. Some studies have found that CD8+ lymphocytes are vulnerable to HIV-1 infection in lung and peripheral circulation (Sadat-Sowti *et al.,* 1994; Bofill *et al.,* 1995; Semenzato *et al.,* 1995; Livingston *et al.,* 1996). However, no HIV-1 p24 antigen was detected in these areas or in the brain tissue during the clinically latent stage, apart from one particular case, subject NA97097, who showed focal p24 positivity in the meningeal lymphocyte infiltration, and the positive cells were confirmed as CD8+ lymphocytes. According to previous studies, HIV does not enter the CNS in a cell free form but rather as a proviral form within an infected cell (Achim *et al.,* 1991). The most likely candidate for the "trojan horse" (Peluso *et al.,* 1985) that carries HIV into the brain is the blood-derived monocyte/macrophage, whereas virus expressing lymphocytes were not observed within the brain (Achim *et al.,* 1991). This unique case that was observed in this study appears to challenge this previous hypothesis. These infiltrating T-lymphocytes did provide some evidence that they might also serve as a carrier of virus into brain tissue. But, again this is a rare event and would require further study in seeking replication of this observation.

Figure 4. 7. P24 immunopositivity was observed in CD8-lymphocytic infiltration area in brain tissue in one particular pre-symptomatic subject (DAB with haematoxylin counter stain. Magnification: x400 (a), x100 (b) & (c), x50 (d) & (e)).

- A typical p24 staining pattern was observed in the meningeal areas with CD8+ lymphocytic infiltration (panel **(a)** & **(c)** demonstrated p24 staining, **(b)** demonstrated CD8 immunopositivity). Cytoplasmic staining was observed within most of the cells (panel **(a)**, as indicated with arrows).
- CD8+ lymphocytic infiltration is an often event within pre-symptomatic individuals in meningeal **(b)** and perivascular (d) areas. In general, these areas within lymphocytic infiltration were p24 negative **(e)**.

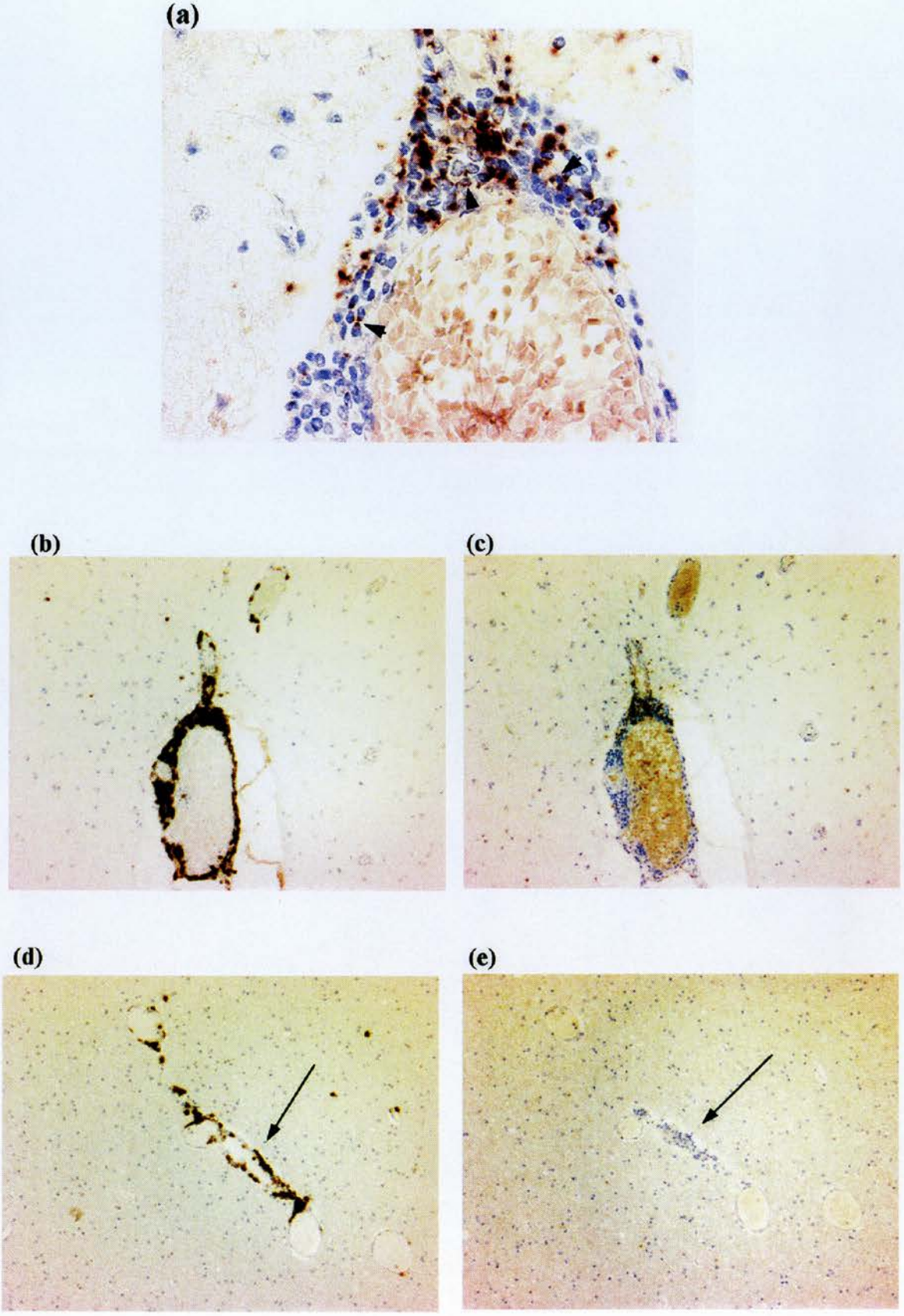


Fig. 4.7

4.4.3 Cellular Localisation and General Distribution of HIV-1 p24 antigen in the Lung Tissue

AIDS-related respiratory diseases have often been reported and usually as the cause of death through bacterial and opportunistic infections. The lung has been reported to be a central organ in HIV-infection, becoming infected perhaps during the asymptomatic period of the disease (Agostini *et al.,* 1996). In general, alveolar macrophages and monocytes within the lung parenchyma and dendritic cells within the airway epithelium are potential APCs, which, together with T lymphocytes, provide the lung with its defense against certain opportunistic and non-opportunistic infections. Pathological examination of the lungs from HIV-1 infected individuals revealed prominent lymphocytic infiltration in lung tissue and formation of lymphoid follicles adjacent to bronchioles.

Previously using PCR technique or in cell culture studies suggested that the majority of lung infections with HIV-1 was in pulmonary macrophages, CD4+ and CD8+ lymphocytes (Agostini *et al.,* 1995; Clarke *et al.,* 1995; Semenzato *et al.,* 1995; reviewed in Agostini *et al.,* 1996). However in this cohort study, HIV-1 p24 antigen detection was restricted to lymphoid follicles and most of the p24 positive cells were morphologically mononucleated. In these follicles that showed p24 immunopostitivity, CD3 and CD68 positive cells were stained and distributed homogeneously, and CD20 positive cells were generally identified around the edge of these follicles. P24 positive cells were morphologically lymphocytes and macrophages. Not all but some mononucleated cells in these areas showed CD21 positivity, and if CD21 could be detected, its staining pattern generally fits the staining pattern of p24 well. Only in one particular case NA91246, a large number of infected multinucleated giant cells, which double-stained with anti-p24 mAb and the PGM1 marker, was observed. Interestingly, no other p24 immunopositivity was detected in other lung blocks taken from this patient, although high levels of macrophage infiltration were also observed. Since most of the studies demonstrated that alveolar macrophages were infected latently (reviewed in Agostini *et al.,* 1995), this case provides the evidence of productive infection within cells of macrophage lineage in lung tissue.

Figure 4. 8. P24 staining patterns in the lung tissue from different study subjects.

- In general, p24 immunopositivity was colocalised in the lymphcytic follicles (**A** to **E**). The staining pattern is similar to that in LN organs (DAB with haematoxylin couter stain **A** to **E**. Magnification: x50, **A** to **C**; x100, **D**; x200, **E**).
- A high level of macrophage infiltration and HIV-1 infected MGCs were observed in one particular case (**F** to **J**), which was demonstrated by double immuno-labelling technique. (DAB for p24 and Vector Red for PGM1 with haematoxylin counter stain. Magnification: x200, **F** & **G**; x100, **H** to **J**). However, another section taken from the same subject was p24 negative (**J**), although a high level of macrophage infiltration was also observed.

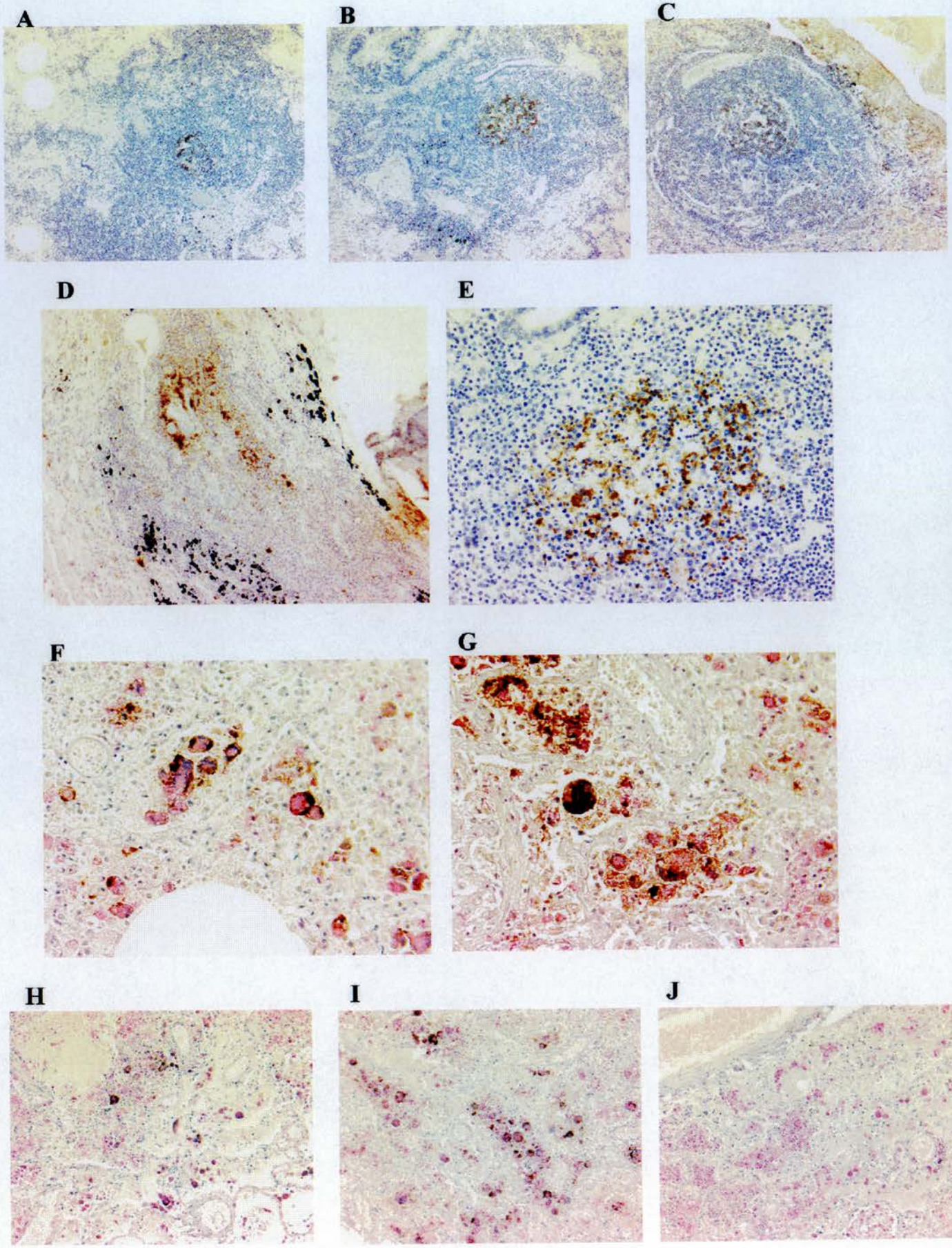


Fig. 4.8

It is difficult to precisely identify the host range of p24 positive cells in lung sections using immunostaining techniques. At present, double-immunolabelling technique was not entirely successful in this tissue. High background and confusing signals highlighted the difficulties of result interpretation. Moreover, in some sections that showed a relatively lower level of p24 positivity, p24 antigen could be detected in one level of the block but further sections generally showed loss of the lymphocytic infiltrated structure, or the p24 immunopositivity disappeared suggesting this was a highly focal event.

4.4.4 Cellular Localisation and General Distribution of HIV-1 p24 Antigen in the Gastrointestinal Tract and Other Organs

HIV-1 proviral DNA has been detected in bowel using PCR technique (Kolter *et al.,* 1991; Donaldson *et al.,* 1994a; van der Hoek *et al.,* 1998), but the p24 positivity in the gut was not often reported. Previous studies have demonstrated that lamina propria might be the major site for HIV infection, most probably in infected macrophages (Kotler *et al.,* 1991; Smith *et al.,* 1997), perhaps because very few CD4+ lymphocytes are present in the GI at the time of bowel symptoms. The frequency of p24 TSA positivity in the present study was relatively low. In this screening detection, the HIV-1 p24 positive staining pattern in gut was similar to that in the lung. HIV-1 p24 antigen was detected rarely, but was restricted to lymphocytic follicles in the bowel. Generally, the p24 positive cells were morphologically mononuclear. As assessed by serial section staining, the p24 positive staining pattern does not match T cells staining, but some of the cells showing abundant cytoplasm and large nucleus are morphologically macrophages. Also, similar to the lung section, if any CD21 positivity could be detected, the p24 staining pattern generally colocalised closely with those CD21 positive FDCs. Moreover, heavy background staining and confusing signal raised by double-labelling technique disturbed the result reading in this tissue. Thus, the precise identification of cellular host of HIV-1 in gut is difficult.

Figure 4. 9. P24 immunopositivity in the ileum.

- Panel A and B demonstrated a serial staining of p24 (A-1), CD21 (A-2), L26 (A-3), PGM1 (A-4) and CD3 (A-5). P24 positive pattern (B-1) was similar with the CD21 positive patter (B-2), although the intensity of CD21 staining was low. (DAB with haematoxylin counter stain. Magnification: x50 for panel A, and x100 for panel B).
- Double immunolabelling of p24 (DAB) and PGM1 (Vector Red) demonstrating that some of these p24 positive cells were macrophages (Magnification: x100, C-1; x400, C-2).

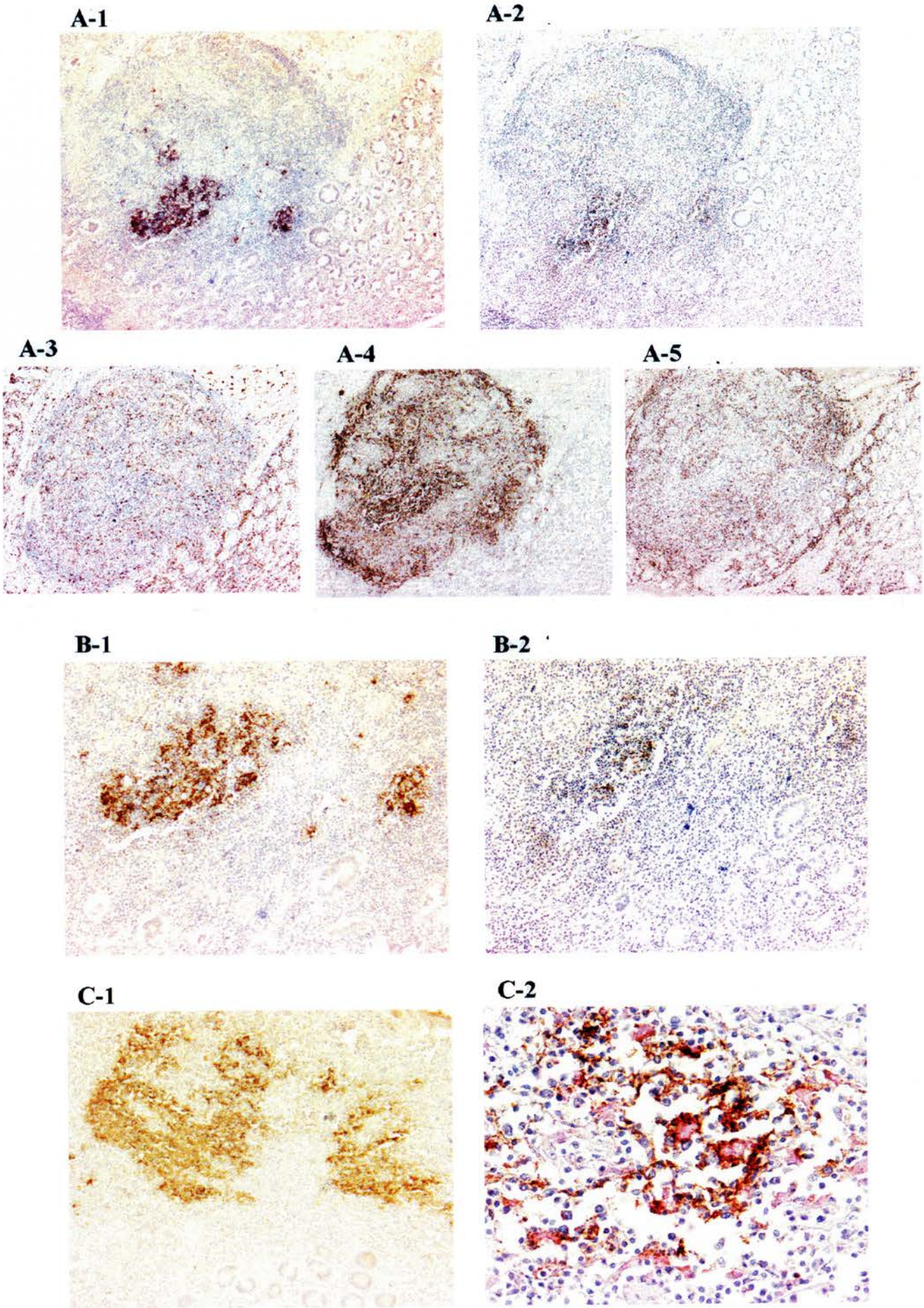


Fig. 4.9

Gastrointestinal dysfunction is prevalent in patients infected with the HIV. Many enteric diseases occur in patients with the late stage of AIDS (Kotler *et al.,* 1991; Sharpstone & Gazzard, 1996; Kearney *et al.,* 1999). It has been reported that the gut-associated lymphoid tissue (GALT) consists of organised lymphoid tissue (Peyer's patches and solitary lymphoid follicles) as well as large numbers of activated memory T lymphocytes diffusely distributed throughout both the intestinal lamina propria and epithelium, and presents as a substantial target for HIV in all phases of infection (Kraehenbuhl, 1998; Kresina & Mathieson, 1999). A recent study in the macaque model has reported a massive CD4+ depletion occurring soon after intravenous simian immunodeficiency virus (SIV) infection (Veazey *et al.,* 1998). Similar to the infection in LN or spleen, the death of these HIV-infected cells resulted in the release of viral particles, which were captured by FDCs as the staining results demonstrated above. These FDC-entrapped viruses could then represent a source for infection as they did in other organs (Kresina & Mathieson, 1999). Unlike lymphoid organs, such an event seems not to occur regularly in the gut, since p24 immunopositivity was not detected often. However, p24 positivity might also be rare because the gut is such a large organ that representative tissues were not sampled regularly.

The HIV-1 p24 antigen was also detected occasionally in salivary gland, liver and kidney, although the frequency was rare and the level of positivity was minor. AIDS-related liver or kidney disease has rarely been reported. In the liver and kidney sections, p24 antigen was rarely identified. When present p24 positive granular staining of occasional cells was restricted to the lymphocyte aggregated areas. Most of the positive cells in the liver appeared morphologically to be sinusoidal macrophages, but a few of them had round nuclei and were CD3 positive, suggesting that they may have been lymphocytes. These few instances of p24 positivity observed in the screening detection in these particular organs could be a result of wide spread virus in the end stages of disease.

4.4.5 General Distribution of CXCR4 and CCR5 Receptors in Various Organs in HIV-1 Infected Individuals

The discovery that infection with HIV required a secondary receptor is an important advance in recent HIV research. Since the first finding in 1996 that CD4+ cells with defective CCR5 receptors from highly exposed but uninfected individuals are resistant to infection with M-tropic isolates (Paxton *et al.,* 1996), this field has continued to undergo rapid progress. Most of the focus was on the usage of chemokine receptor and the interaction between viral envelope proteins and coreceptors (reviewed in Broder & Collman, 1997; Moore, 1997). There were few articles demonstrated the localisation of HIV-1 co-receptors in the brain (Vallat *et al.,* 1998; Zhang *et al.,* 1998), intestine (Zhang *et al.,* 1998; Dwinell *et al.,* 1999) and other tissues (Zhang *et al.,* 1998).

In this study, immunohistochemistry staining was employed to investigate the general distribution of chemokine receptors, CXCR4 and CCR5. In general, the staining pattern of both antibodies was diffuse and cytoplasmic, and the distribution of these two receptors showed overlap. They picked up similar cell populations but the staining of CXCR4 was more intense than CCR5 in general.

Although these two molecules have been found recently to be co-receptors for HIV-1 entry, the IHC staining pattern is complicated. Generally, in the brain tissue, neurons are the only cell type consistently stained (Figure 4. 10). Other cells of neuroectodermal origin and blood vessel endothelial cells were stained occasionally. In some brain sections, most of the cells, including microglia, astrocytes and oligodendrocytes were CXCR4 and CCR5 positive, however, in other sections, immunopositivity was only observed in neurons. In spleen and LNs, both antibodies appear to localise in cells with round nuclei and abundant cytoplasm, which are located scattered outside the follicles. These positive cells are morphologically of macrophage and lymphocytic appearance. Also, endothelium cells are occasionally stained. However, none of the cells inside the follicles was stained.

Figure 4. 10. Immunohistochemical localisation of CXCR4 (A to F) and CCR5 (G & H) receptors in the brain tissue (DAB with haematoxylin counter stain. Magnification: x50, A to C; x200, D to f; x100, G & H).

- In general, neuron is consistently stained with CXCR4 (**A** and **D**) and CCR5 (**G**).
- The staining patterns of cells of neuroectodermal origin were various. In some cases, most of the cells showed CXCR4 (**B** & **E**) and CCR5 (data not shown) negative. However, in other cases, most of the cells were CXCR4 (**C** & **F**) and CCR5 (**H**) positive.

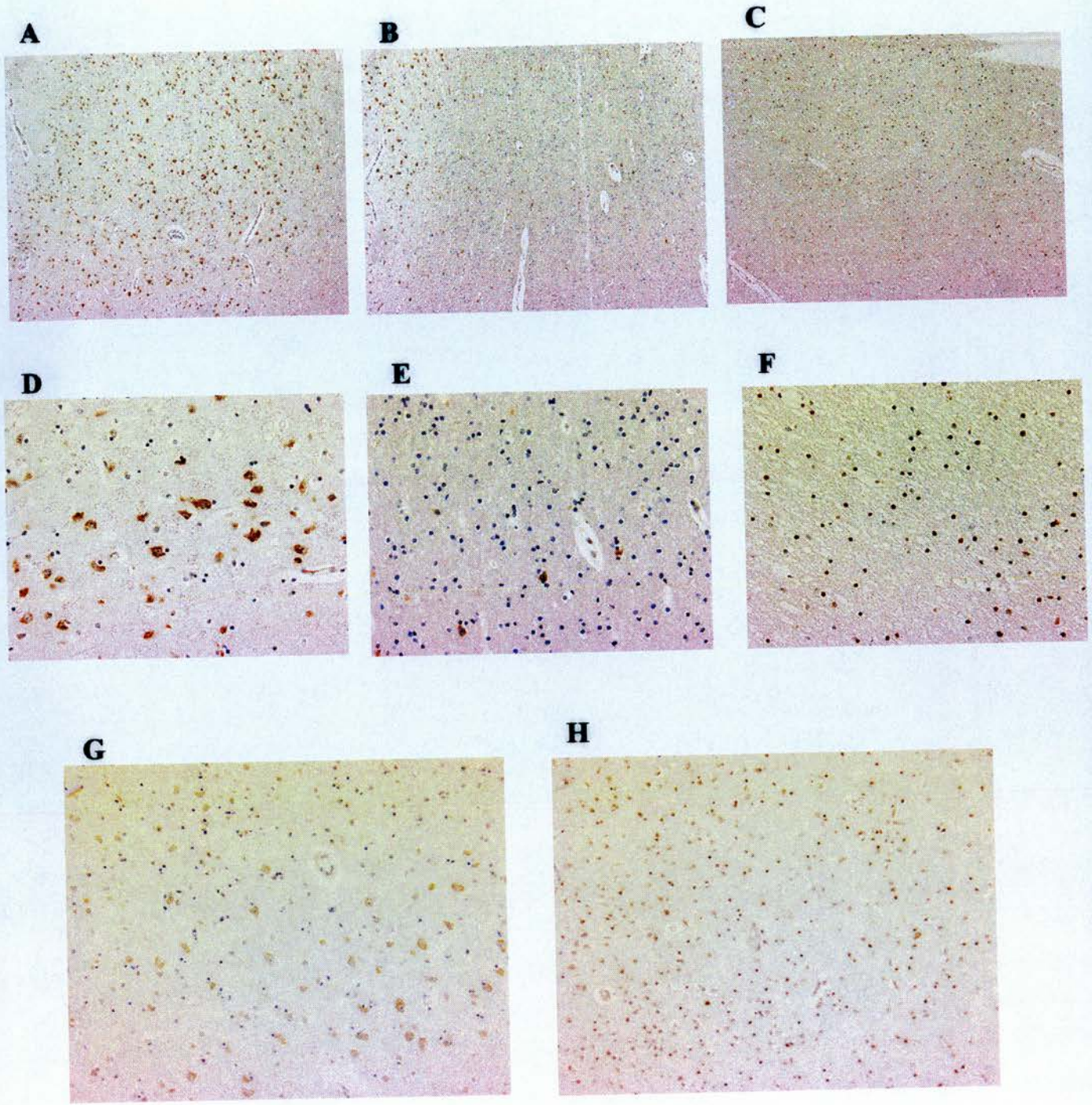


Fig. 4.10

Figure 4. 11. Immunohistochemical localisation of CXCR4 receptor in the spleen (A & B), lung (C & D) and Ileum (E & F). (DAB with haematoxylin counter stain. Magnification: x100).

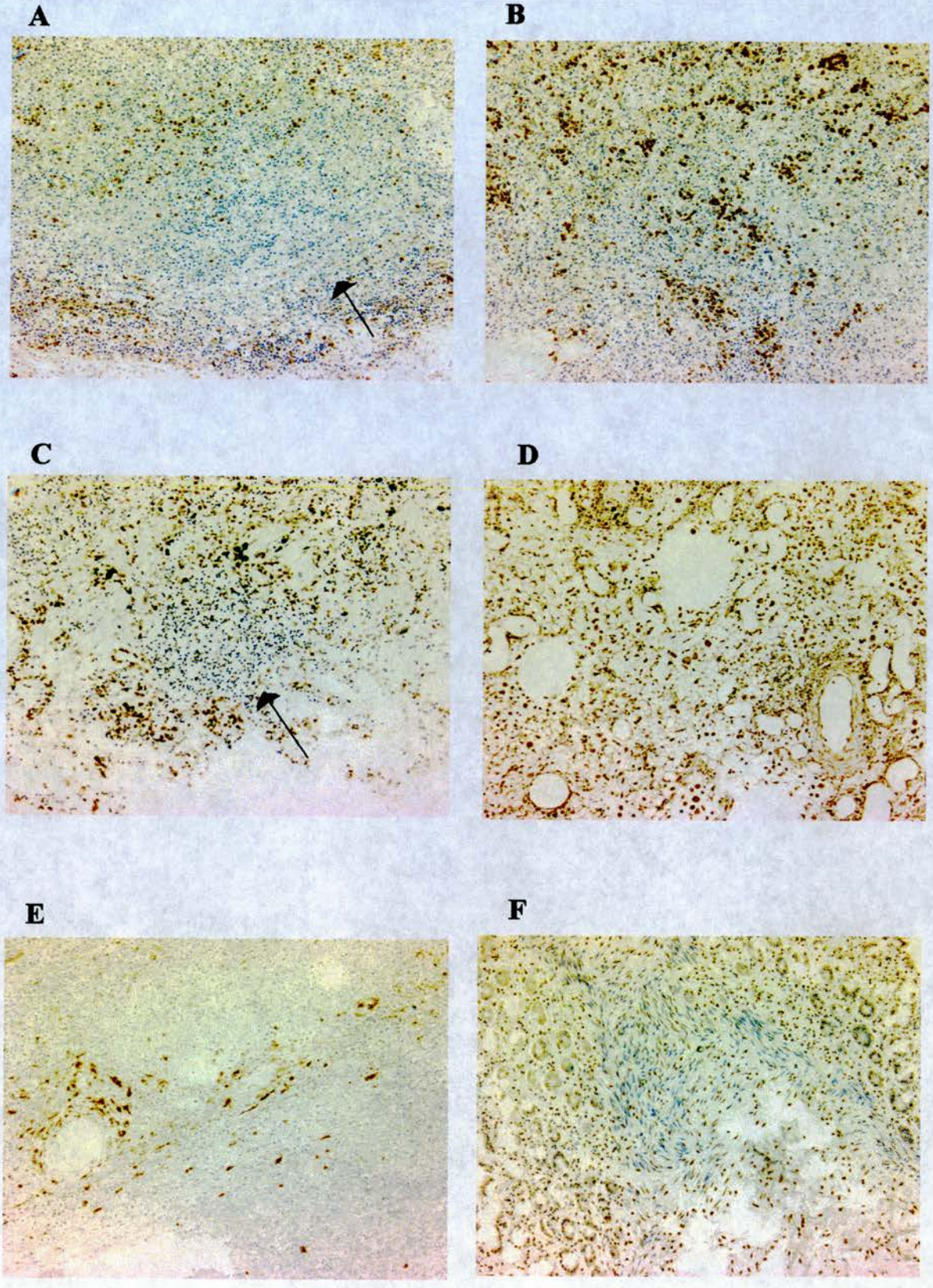


Fig. 4.11

In lungs and intestine, a high level of CXCR4 and CCR5 was visualised in epithelial cells, blood vessel endothelial cells, tissue macrophages and lymphocytes. Interestingly, in the lymphoid aggregates or lymphoid follicles where p24 antigen was generally detected, very low level of CXCR4 nor CCR5 expression was detected. No correlation was observed between the distribution of CXCR4 and CCR5, and HIV-1 p24 detection in the present study. However, these staining results were not quite reproducible. Whether this lack of correlation resulted from the use of less than optimal antibodies to chemokine receptors or reflects the likelihood that the *in vivo* mechanisms are more complex requires further investigation.

4.4.6 Limitations of The Detection Methods

Although the application of TSA in p24 IHC detection did improve the staining results, there were still some limitations to the technique. One problem is the application of the double immunolabelling technique. The purpose of this study was not only viral detection but also the identification of the cellular host of HIV-1, and the best technique should be double-labelling. Several technical problems have occurred while applying this technique, such as different pretreatments required for the two working antibodies, cross-reaction because of the antibodies raised in the same species, and also in balancing the intensity of the two positive signals. The problem of pretreatment was particularly acute when working with CD21 and p24. CD21 mAb is extremely heat-resistant and requires protease for antigen retrieval, but p24 could not be stained without heat-pretreatment. Most of the sections failed to adhere to the slides during staining because of the application of the two strong antigen retrievals. The serial section assay was applied to resolve this problem, but the results were difficult to correlate because staining was performed in different sections. Also, the recombination of the colours in end point detection was a problem, since most of the time, the two colours were mixed causing confusion in reading the results. Double immunofluorescence has also been employed. However, the different antigen retrievals, and cross-reaction between antibodies limited the

success of this technique.

Sampling is another problem, which has been highlighted in some autopsy cases. For example, in patient 96-160, two spleen tissue blocks were taken for examination. One showed strong p24 positive staining, which the other was completely negative. Further evidence was found in non-lymphoid tissue blocks, where p24 antigen was detected in the first few sections taken, but as the block was examined at deeper levels, the p24 positive areas disappeared. Therefore, the localisation and the level of viral activity in large organs, such as intestine and lung, were difficult to determine. Some important information might be omitted. Within these organs, multisampling is indispensable.

Moreover, a problem that might contribute to negative results is tissue fixation, especially in tissue blocks which had been fixed in formalin for several weeks. As described in section 3.1, formalin fixation preserved the best morphology for histological examination; on the other hand, it decreased the sensitivity of IHC detection. For some antibodies, such as CXCR4 and CCR5 that did not work consistently, short-fixed samples were required.

Also, the interpretation of negative results is difficult. HIV-1 p24 protein is a viral structural protein, which is only expressed during productive infection. A different technique will be required to investigate the frequency and distribution of latently infected cells. According to other *in vitro* studies (refer to section 4.1), HIV-1 is able to infect several different cell lines non-productively without expression of structural proteins. In addition, the advent of HAART, which has been found to suppress viral replication *in vivo* very considerably, might have predisposed to some negative results found in some recent cases.

To investigate further, frozen tissues from the nine recent autopsies were subjected to molecular analysis. Details of the molecular information and the basis for comparison between histopathologic and genetic information is described in chapter 5.

Chapter 5: Genetic Characterisation of HIV-1 Infecting the Lymphoid and non-lymphoid organs in Different Stages of Infection

5.1 Introduction

A noticeable feature of HIV-1 is the extraordinary diversity of the viral genome. Usually, an HIV-1 infected individual harbours a swarm of closely related but genetically distinct viruses, which comprise the HIV quasispecies. These HIV-1 variants demonstrate great heterogeneity in their biological properties, such as syncytium-inducing capacity, replication rate, and cellular tropism (Cheng-Mayer *et al.,* 1988; Fenyo *et al.,* 1988; Tersmette *et al.,* 1989a; Tersmette *et al.,* 1989b). It is believed that differences in these properties may contribute to the pathogenesis of HIV-1 infection.

An apparent redistribution of virus upon disease progression was observed to occur at the same stage of disease as the change from a non-syncytium-inducing (NSI) to a syncytium-inducing (SI) phenotype. During the pre-symptomatic stage of infection, a highly restricted distribution of HIV-1 was confined to cells of the lymphoid system (Donaldson *et al.,* 1994a). HIV-1 isolates from patients in this stage were relatively homogeneous, more slowly replicating, preferentially macrophage-tropic NSI strains (Schuitemaker *et al.,* 1992; Zhu *et al.,* 1993; van't Wout *et al.,* 1994; Moore, 1997). With the development of symptomatic disease, proviral DNA was widely recovered from both lymphoid and non-lymphoid organs (Donaldson *et al.,* 1994a). HIV-1 isolates from these patients who died of complications associated with AIDS were more likely to be rapidly replicating, preferentially T cell tropic SI strains (Zhu *et al.,* 1993; Coffin, 1995; Moore, 1997). Moreover, the emergence of SI variants has been reported to precede a rapid decline of CD4+ lymphocytes in the peripheral circulation and a more rapid onset of AIDS (Keet *et al.,* 1993; Koot *et al.,* 1993; Karlsson *et al.,* 1994). Thus, the wide spread of viruses throughout the body, the switch in virus phenotype from NSI strains to SI strains, together with CD4+ cell numbers and virus load were thought to be the important prognostic markers of disease progression.

The possible determinants which governed the SI capacity and cell tropism

were presumed to lie within the hypervariable regions (V1 to V5) of the HIV-1 *env* gene, particularly in the V3 region (Shioda *et al.,* 1991; Groenink *et al.,* 1992; Donaldson *et al.,* 1994b; Broder & Collman, 1997). The V3 domain contains 35 amino acids arranged in a disulfide loop involving Cys301 and Cys336, and serves as a major target for neutralizing antibodies (LaRosa *et al.,* 1990; Robert-Guroff *et al.,* 1994). This domain has been presumed to be important for HIV-1 escaping from antibody neutralization or cytotoxic T cell elimination (LaRosa *et al.,* 1990; Barré-Sinoussi, 1996) (also refer to section 1.4). Sequence comparisons have demonstrated considerable diversity in the V3 region. Usually, nonsynonymous nucleotide substitutions, which resulted in a change of an amino acid, were more frequent than synonymous ones which did not lead to a change of the amino acid within the V3 domain. Some of these nonsynonymous substitutions might be related to resistance to neutralization, some might be related to antibody enhancement, and certain amino acid substitutions in the V3 domain were associated with the appearance of SI virus strains (Lukashov *et al.,* 1995). For example, analysis of natural variants of HIV-1 indicated that basic amino acids in one or more of positions 11, 24, 25 and 32 (from the first cysteine) confer a SI phenotype, whereas neutral or negatively charged amino acids in these positions correspond with a NSI phenotype (Chesebro *et al.,* 1992; de Jong *et al.,* 1992; Fouchier *et al.,* 1992; Milich *et al.,* 1993). Generally, the V3 sequences from SI isolates presented a broad range of substitutions, insertions, and deletions at most positions between the disulfide-bridged cysteine residues of the putative V3 loop; in contrast, those from NSI isolates showed relatively less diversity (Donaldson *et al.,* 1994b). Moreover, NSI/macrophage tropic isolates are commonly associated with a lower charge by calculation of the overall net charge of the V3 sequence between the disulfide-bridged cysteine residues, while SI/ T cell tropic isolates were associated with higher charge (Milich *et al.,* 1993; Donaldson *et al.,* 1994b).

A wide range of V3 sequence variants was recovered from various organs from AIDS patients but only from lymphoid organs from pre-AIDS individuals (Donaldson *et al.,* 1994a). A general observation was a common set of V3 sequences being presented at varying frequencies throughout the body; however, there were

distinct V3 populations existing in different organs (Donaldson *et al.,* 1994b). Whether the V3 domain operated as the principle determinant of tissue tropism was uncertain. Some researchers suggested that the consistent sequence differences in the V3 region provided indirect evidence for specific cellular tropisms (Hwang *et al.,* 1991; Ball *et al.,* 1994; Korber *et al.,* 1994; Reddy *et al.,* 1996); however, others suggested that these sequence variants merely represented the differences in the rate of virus turnover in different organs (van der Hoek *et al.,* 1996; van der Hoek *et al.,* 1998).

Genetic diversification in the V3 and $p17^{gag}$ regions occurs under different selection pressures. Unlike the V3 region, where nonsynonymous substitutions are commonly observed, most nucleotide differences in $p17^{gag}$ region appear to be synonymous (Kasper *et al.,* 1995). In contrast to nonsynonymous substitutions that may arise in escape mutants through selection pressures exerted by the host immune system, synonymous substitutions do not cause changes of amino acids and are therefore less subject to positive selection pressures. As synonymous substitutions are more likely to be caused by a random fixation of mutations (neutral theory), they presumably reflect the underlying mutation rate, allowing reliable estimates of divergence times to be calculated (Holmes *et al.,* 1993). The rate of sequence changes in the $p17^{gag}$ region has previously been determined from haemophiliacs in a Bonn cohort (Kasper *et al.,* 1995). As a constant rate of synonymous substitution was observed in $p17^{gag}$ across evolutionary time, this region was proposed as a useful marker for constructing epidemiological relationships between HIV-1 infected individuals (Kasper *et al.,* 1995). These synonymous changes in $p17^{gag}$ can also be used as a type of molecular clock allowing estimates of the divergence time between any two sequences to be carried out, and can be extended to the comparison of variants within a single infected individual (Hughes *et al.,* 1997).

Studies in genomic diversity have also suggested that recombination of HIV-1 genomes may play a role in producing new viruses with different biological and pathological properties (Robertson *et al.,* 1995). This recombination of viral genomes could also introduce new strains into the population and thereby challenge

vaccine approaches to HIV. The extent of recombination between strains is not clear, but it is believed to be a frequent event during the course of infection (Robertson *et al.,* 1995), and appears to occur most often within the *gag* and *env* regions (Cornelissen *et al.,* 1996; Kampinga *et al.,* 1997).

To investigate the association between virus distribution, phenotype, nucleotide sequence diversity and the progression of disease, this study was carried out using the nested PCR limiting dilution and nucleotide sequences in V3 and $p17^{gag}$ regions from various organs obtained at autopsy.

5.2 Materials and Methods

5.2.1 Study Subjects

Frozen samples of lymph node (LN), lung, colon, and left frontal lobe of brain (LF) were obtained from nine HIV-1-infected autopsies, including six symptomatic individuals (NA96425, NA97021, NA97020, NA97017, NA96272 and NA96371) and three pre-symptomatic individuals (NA98025, NA98028 and NA97097). The patients had died shortly before this study commenced. Most of them were in the risk group of intravenous drug user, excluding NA96371 and NA97020 who were male homosexuals. Their ages ranged from 32 to 49, and with a history of HIV-1 infection lasting 4 to 13 years before death. General information for each study subject is listed in Table 5. 1. For details of the pathological findings refer to appendix II.

DNA from each sample was extracted according to the protocol described at section 2.6. Total DNA concentration was estimated by spectrophotometer at 260nm. One microgram aliquots of extracted DNA were amplified in the V3 and $p17^{gag}$ regions using primers as previously described (refer to section 2.7). PCR amplified DNA was then used for further molecular investigations.

Table 5. 1. Clinical information for study subjects.

Study Subject	Gender / Age	Risk Group[a]	Yrs since first HIV-positive[b]	CD4[c]	CD8[c]	Rx[d]	AIDS-Related Illness[e]
NA96425	M/32	ADM	12	1	298	No	No AIDS-related illness (AIDS was diagnosed on the CD4 counts alone)
NA96371	M/34	AH	4 (2.5)	3	100	Yes	KS
NA97020	M/49	AH	6 (2.5)	8	388	Yes	MAI
NA97017	M/32	ADM	11 (1.5)	16	120	Yes	PCP
NA96272	M/32	ADM	9	126	636	No	No AIDS-related illness (AIDS was diagnosed on the CD4 counts alone)
NA97021	F/49	ADM	12 (4)	137	1620	Yes	HIVE
NA98025	M/43	P-Hetero	6	297	323	No	Clinically in Pre-AIDS
NA98028	F/48	PDM	13	703	4091	No	Clinically in Pre-AIDS
NA97097	M/36	PDM	11	NA	NA	NA	Clinically in Pre-AIDS

Key for Table 5. 1:

[a]: Risk Groups: ADM --- AIDS Drug Misuser; AH --- AIDS Homosexual;
PDM --- pre-AIDS Drug Misuser; P-Hetero --- pre-AIDS heterosexual

[b]: The years of patient living with HIV, the number in the brackets indicates the number of years between the first AIDS presenting illness and death.

[c]: CD4, CD8: The last CD4 counts and CD8 counts

[d]: Rx: Antiretroviral Therapy. Details of the history of treatment are listed in appendix II

[e]: MAI --- *Mycobacteria avium-intracellulare*;
PCP --- *Pneumocystis carinii* pneumonia;
KS--- Kaposi's Sarcoma;
HIVE --- HIV encephalitis.

5.2.2 Estimation of Viral Load

Quantitation of proviral DNA was carried out using the PCR limiting dilution method. The number of copies of provirus per million cells was determined by nested PCR amplification of serial ten-fold dilutions of DNA using the $p17^{gag}$ primers. Ten replicates at last positive dilution were used to indicate the minimum proviral load in the sample, assuming a Poisson distribution for each sample by $-\ln(1-p)/d$ (where p= proportion of positive samples and d= dilution). Viral load was expressed as copies per million cells on the basis that a human diploid cell contains 6.6pg DNA.

5.2.3 Genetic Analysis

PCR amplified DNA was cloned into pGEM-T vector using poly (T) overhangs (refer to section 2.8). Proviral DNA from clones was sequenced using the Sequenase version 2.0 kit (USB) following the manufacturers' protocol (refer to section 2.7). The sequence dataset for the $p17^{gag}$ region extended from positions 405-795 in the HIV_{LAI} genomic sequence (GenBank accession number K02013), while the V3 sequences were compared from positions 7017-7322. Sequences were then aligned and diversity estimated using the Simmonic 2000 Sequence Editor package. Unless shown otherwise, ten clones from each of the positive samples were sequenced. Phylogenetic analysis was carried out using MEGA program. Distributions of pairwise distances were plotted using the Systat statistical package.

$p17^{gag}$ and V3 sequences from each of the study subjects were monophyletic upon comparison with sequences from other individuals in the study, from previously described HIV-infected individuals in Edinburgh, and from the following published sequences of clade B isolates: HIVSF2 (K02007), HIVRF (M17451), HIVOYI (M26727), HIVLAI (K02013), HIVJRFL (M74978), HIVYU2 (M93258), HIVCAM1 (D10112), HIVNY5CG (M38431), HIVHAN (U43131), HIVWMJ22 (M12507), and HIVSFAAA (M65024). This comparison provided no evidence for inter-sample or exogenous laboratory contamination.

5.3 Results

5.3.1 HIV-1 *in vivo* Distribution and Sequences Variability in Various Organs Obtained from Presymptomatic and Symptomatic Autopsies

Two regions of HIV-1 proviral DNA, $p17^{gag}$ and V3 were successfully amplified by nested PCR from all LN and lung samples, and from five colon samples (NA97021, NA97017, NA96425, NA96272 and NA98025), and five LF samples (NA97021, NA97020, NA96371, NA98025, NA97097). Proviral DNA quantification, and the diversity of cloned sequences was estimated as described in section 5.2.2 and section 5.2.3. Estimated viral load and sequence diversity was listed in Table 5. 2.

Proviral load varied considerably between individuals (Table 5. 2). In general, high levels of HIV-1 proviral DNA were consistently detected in the lymph nodes and lungs, except in patients NA97017 and NA96272 in whom low viral load was detected throughout the four examined organs. In contrast, the presence of proviral DNA in samples from colon and brain tissues was only within a minority of symptomatic individuals.

No association between the detection of HIV-1 proviral DNA and the disease progression or risk groups was observed within samples from LN or lung. However, the detection of HIV-1 proviral DNA in the brain demonstrated a close correlation with the pathological appearance of HIV encephalitis (HIVE). This was observed within subjects NA97021, NA97020 and NA98025, but not in NA96371 or NA97097 in whom low levels of proviral DNA were detected in the brain without evidence of HIVE. As for the colon samples, a high level of proviral DNA was generally found within symptomatic subjects, although no specific pathology appearance was observed.

Keys for table 5.2:

Study subjects are listed in the first column according to the last CD4+ cell counts with the lowest CD4 counts at first.

[a] Load: Proviral load is quantified by limiting dilution using nested primer in the $p17^{gag}$ regions. Viral load was expressed as copies of proviral DNA per 10^6 cells

[b] Het: Intra-population sequence heterogeneity (mean pairwise p-distance) in V3 (above) and $p17^{gag}$ (below) regions.

[c]: Phen: Inferred phenotype of virus from V3 sequence. X4 represents CXCR4 dependent; R5 represents CCR5-dependent.

[d]: p24: immunohistochemical detection of p24 antigen in tissues. Results are collected from chapter 4.

[e]: HIVE: HIV-encephalitis is diagnosed according to the presence of giant cells and/or p24 immunopositivity in the brain tissue

[f]: less than 0.5 copies per 10^6 cells

[g]: HIV positive man with probable incipient HIV encephalitis (one giant cell is identified).

[h]: p24 antigen was detected only within the lymphocytic infiltrate of leptomeninges, and within CD8 positive cell group (Refer to section 4.4).

Underlined samples: the observed heterogeneity was within the range introduced by *Taq* error.

Shaded areas: pre-symptomatic individuals

ND: not done

Table 5. 2. Summary of quantitation, phenotype and heterogeneity of HIV-1 in different tissues of 9 study subjects

Study Subject	Lymph Node				Lung				Colon				Brain Left Frontal				
	Load[a]	Het[b]	Phen[c]	p24[d]	Load	Het	Phen	p24	Load	Het	Phen	p24	Load	Het	Phen	p24	HIVE[e]
NA96425	11000	0.0258 0.0214	X4/R5	1+	10500	0.0093 0.0205	R5	+	1400	0.0123 0.0097	X4	0	<0.5[f]	ND	ND	0	No
NA96371	6000	0.0331 0.0271	R5/X4	1+	900	0.0315 0.0244	R5/X4	0	< 0.5	ND	ND	0	30	0.0216 0.0026	X4	0	No
NA97020	90000	0.0458 0.0212	X4	0	600	0.0059 0.0234	X4	0	ND	ND	ND	0	6	0.0124 0.0036	X4	1+	**Yes**
NA97017	15	0.0043 0.0038	R5	0	10	0.0504 0.0137	R5	0	10	0.0058 0.0168	R5	0	<0.5	ND	ND	0	No
NA96272	50	0.0148 0.0029	R5	1+	50	0.0213 0.0273	R5	0	20	0.0403 0.0338	R5	2+	<0.5	ND	ND	0	No
NA97021	110000	0.0108 0.0180	R5	3+	60000	0.0209 0.0161	R5	2+	11000	0.0424 0.0331	R5	0	20000	0.0175 0.0397	R5	3+	**Yes**
NA98025	150	0.0266 0.0320	R5	3+	1500	0.0178 0.0390	R5	0	65	0.0378 0.0240	R5	2+	100	0.0455 0.0221	R5	0	**Yes**[g]
NA98028	60	0.0146 0.0111	R5	3+	60	0.0094 0.0248	R5	0	<0.5	ND	ND	0	<0.5	ND	ND	0	No
NA97097	110000	0.0272 0.0272	R5	3+	14000	0.0072 0.0117	R5	0	<0.5	ND	ND	ND	3	0.0087 0.0041	R5	1+[h]	No

Each PCR amplified DNA was cloned and ten clones from each library sequenced. Mean intra-sample pairwise p distances ranged from 0.0038 to 0.0504 in the V3 region, and from 0.0026 to 0.0397 in $p17^{gag}$ (Table 5. 2). Amongst the 28 PCR-positive samples, the observed heterogeneity within eight (underlined samples indicated in Table 5. 2) was within the range introduced by *Taq* errors during template amplification. Assuming a frequency of 0.5×10^{-4} misincorporations per base, and 40 effective copying cycles during nested PCR, the expected diversity resulting from *Taq* error is 0.004 (Saiki *et al.*, 1988; Ennis *et al.*, 1990; Leigh Brown & Simmonds, 1995). The possibility that nucleotide sequences derived from a single template was supported by the association between low sequence diversity (within the range of *Taq* error) and low virus load in these samples. For example, the estimate for virus load in the brain sample from NA97097 was 3 copies per 10^6 cells, calculated from the frequency of 4 positive reactions from testing 10 replicate one μg aliquots of DNA with the $p17^{gag}$ primers. As one μg amounts of DNA were used for sequencing, it is highly likely that each of the cloned sequences ultimately derived from a single proviral copy. Moreover, these sequences derived from a single proviral copy were monophyletic and clustered as a unique lineage, which gives rise to a misleading impression of tissue specificity.

This problem of single templates is likely to prevent estimates of true population diversity being made, and to generate mis-interpretations of the genetic relationships between virus variants that isolated from various organs (Leigh Brown & Simmonds, 1995). Furthermore, as described in a previous study (Donaldson *et al.*, 1994a), it was impossible to determine whether the sequences obtained from samples with low virus loads derived from the tissue itself or from residual blood. For more accurate analysis of the genetic relationships between these virus isolates, sequence data should be examined carefully before they are subjected to phylogenetic or evolutionary analysis. Those samples showing little diversity together with low viral load have therefore been excluded from the subsequent analysis.

Intra-sample sequence diversity in the $p17^{gag}$ region correlated with that in the V3 region (Figure 5.1: Spearman rank correlation coefficient 0.31; p=0.085). No association was observed between the sequence variability and risk groups, disease progression or any known risk factors. However, samples from non-CNS organs, including lung, colon and LF commonly showed greater sequence diversity than lymph node, although this difference was not significant.

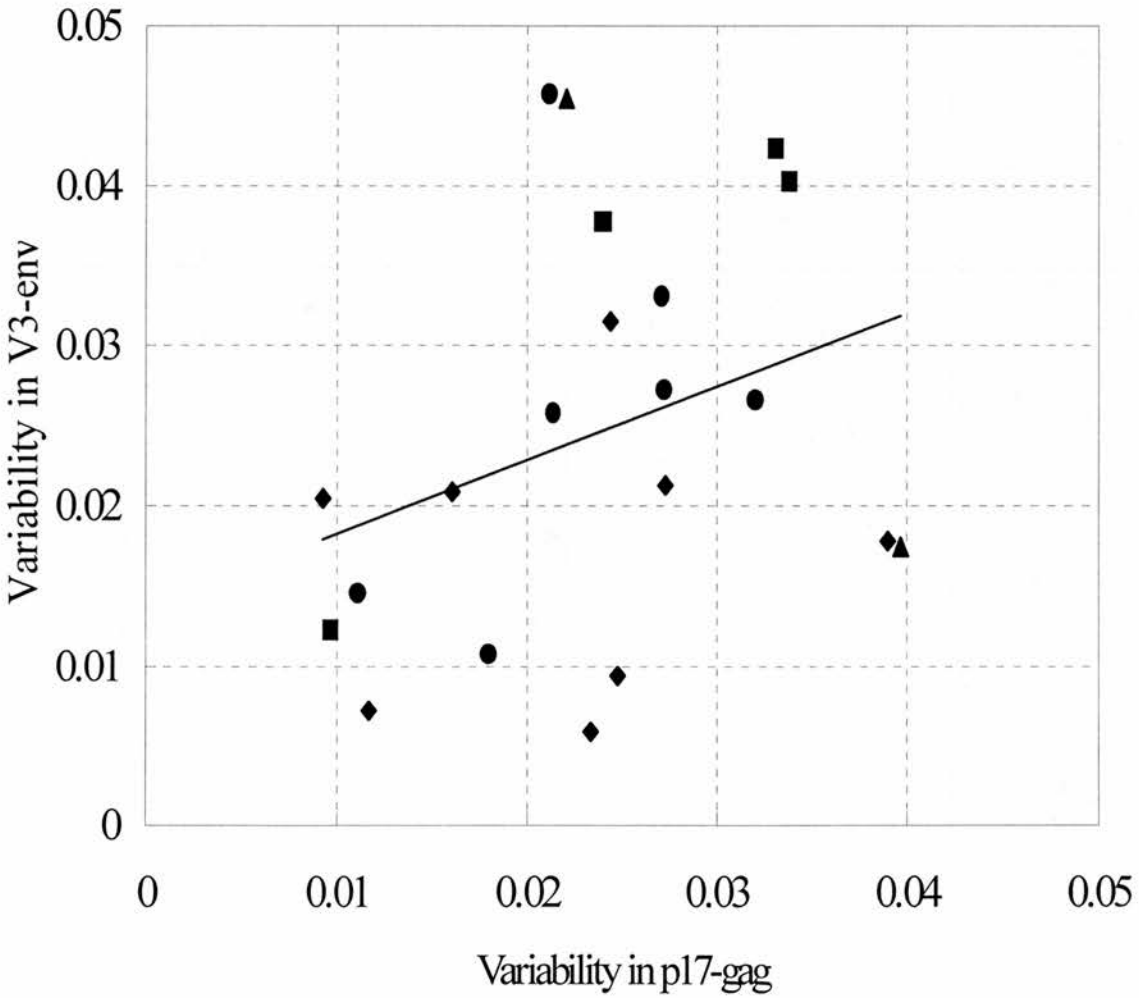


Figure 5. 1. Intrasample sequence diversity in the $p17^{gag}$ and V3 regions of LN (●), lung (◆), colon (■) and brain (▲) from nine study subjects (Spearman rank correlation coefficient: 0.31; p=0.085).

5.3.2 Prediction of Viral Phenotype

In this study, viral phenotype was predicted according to the translation of the nucleotide sequences between positions Cys301 and Cys336 in the V3 region. This is the method commonly used for phenotype prediction directly from sequences. In general, if the positively charged amino acids arginine (R) or lysine (K) were observed at positions 11 or 25 counted from the first cysteine (Cys301) in the V3 loop, variants were predicted as SI strains, otherwise they were predicted as NSI strains. In general, SI strains were also known as T cell tropic using CXCR4 as co-receptor whereas NSI strains are preferentially macrophage-tropic and use CCR5 as co-receptor. Details of the V3 amino acid sequence of each sample are listed in Figure 5. 2).

Most variants contained neutral amino acids at position 11 and acidic residues at position 25, and were predicted as CCR5-dependent (NSI) strains. Variants recovered from NA96371 and NA97020 (LN and lung) and from NA96425 (LN and colon) which contained basic amino acid substitutions at position 11 were identified as CXCR4-dependent (SI) strains. The presence of CXCR4-dependent strains correlated well with disease progression. In general, CXCR4-dependent variants were found preferentially in sympomatic individuals, in whom low CD4 cell counts were found at death (NA96425, NA96371 and NA97020), and in the male homosexual risk group (NA96371 and NA97020). In contrast, all of the variants isolated from pre-symptomatic subjects (NA97097, NA98025, and NA98028) and from those with higher CD4 cell counts at death (NA97021 and NA96272) were identified as CCR5-dependent strains.

Alternatively, the viral phenotype could be predicted according to the overall net charge and the similarity to the subtype B consensus within V3 loop. Previously, the net charge and the number of amino acid changes within V3 sequences were calculated from 59 isolates with known phenotype *in vitro* (Donaldson *et al.,* 1994b). Most of the NSI/macrophage isolates were consistently associated with lower net charge and higher similarity with consensus sequence, and the SI/T cell tropic strains were commonly associated with higher net charge but were more diverse. A

diagnostic line (y=-5x+25; x: overall V3 charge; y: number of amino acid changes) was then designed, which clearly separated the known NSI and SI populations (Donaldson *et al.,* 1994b). To further investigate whether or not the method they designed could also be applied to the variants which are directly recovered from tissues, the sequence data obtained in this study were also analysed using the same parameters. The overall net charge was calculated by assigning a unitary negative charge to each aspartic acid (D) and glutamic acid (E) and a unitary positive charge to each lysine (K) and arginine (R) between the two cysteine residues. The similarity was expressed as the number of amino acid changes compared to the consensus sequence (Table 5. 3 & Figure 5. 2).

The results of viral phenotype prediction using the two different methods were similar (Table 5. 3). Those predicted as SI strains are primarily associated with higher net V3 charge and less similarity to the subtype B consensus. For example, in patient NA96425, most of the sequences containing basic amino acid substitutions at position 11, which predicted them as SI strains, were associated with a net charge higher than 4, and had at least 7 amino acids different from the consensus, and were commonly located to the right of that diagnostic line. In contrast, predicted NSI strains show lower charge and greater similarity to the subtype B consensus, as observed in patient NA98028, whose isolates were generally found to have a charge lower than 3, showed no difference to consensus V3 sequences, and were located to left of diagnostic line. At present, most groups predict viral phenotype according to the translation of the nucleotide sequences between positions Cys301 and Cys336 in the V3 region. The results demonstrated here suggest that a system involving net charge and amino acid changes within the V3 loop could also be used as a predictive method.

Table 5. 3. Comparison of viral phenotype prediction in lymph node, lung, colon and LF brain tissues using two different predicting systems. Study subjects are listed in the first column according to the last CD4+ cell counts with the lowest CD4 counts first.

	LN	LU	CO	LF
NA96 425	X4 (5/9); R5 (4/9)[a] **X4 (8/9); R5 (1/9)*[b]*	R5 ** R5 (5/8); X4 (3/9) **	X4 **X4 (12/13);R5 (1/13)**	N/A
NA96 371	R5 (8/12); X4 (4/12) ** R5 (8/12); X4 (4/12)**	R5 (9/12); X4 (3/12) **R5 (10/12); X4 (2/12)**	N/A	N/A
NA97 020	X4 (10/10) **R5 (9/10); X4 (1/10)**	X4 **R5**	N/A	N/A
NA97 017	R5 **R5**	R5 **R5**	N/A	N/A
NA96 272	R5 **R5 (9/11); X4 (2/11)**	R5 **R5 (8/10); X4(2/10)**	R5 ** R5 (8/10);X4 (2/10)**	N/A
NA97 021	R5 **R5**	R5 **R5**	R5 ** R5 (7/9); X4 (2/9) **	R5 **R5 (5/10);X4 (5/10)**
NA98 025	R5 **R5(7/9);X4(2/9)**	R5 **R5**	R5 ** R5 (9/10);X4 (1/10)**	R5 **R5 **
NA98 028	R5 **R5**	R5 **R5**	N/A	N/A
NA97 097	R5 **R5 **	R5 **R5**	N/A	N/A

Keys for Table 5. 3:

[a] Prediction according to the amino acid at positions 11 and 25.

[b] Prediction according to the diagnostic line (Donaldson *et al.*, 1994b).

R5: CCR5-dependent (NSI); X4: CXCR4-dependent (SI). The figure in brackets represents this strain as a fraction of the total.

N/A: no data available (the viral load was less than 0.5 per 10^6 cells, or the diversity was within *Taq* error).

Shaded-area: pre-symptomatic individuals

Figure 5. 2. List of inferred amino acid sequences of variants from the V3 region of (a) NA96425, (b) NA96271, (c) NANA97020, (d) NA97017, (e) NA96272, (f) NA97021, (g) NA98025, (h) NA98028, (i) NA97097, using the LN variants as a reference. The organs that were examined are divided by horizontal lines. Amino acids lying between two invariant cysteines at position 303 and 338 are blocked and compared with North American and European Consensus sequences (LaRosa *et al.*, 1990). Sequences are numbered by their position of the HIV_{LAI} gp120 sequence. The actual number of clones sequenced is listed in column "Clone". The net charges within two cysteines, which were calculated by assigning a unitary negative charge to aspartic acid (D) and glutamic acid (E) and a unitary positive charge to lysine (K) and arginine (R) are listed in column "*x*". The number of amino acid changes from the consensus sequence are listed in column "*n*". The predicted NSI (N) or SI (S) strains are listed in column "P".

Symbols "**.....**" represent the sequence identity with reference sequences;

Symbols "**-**" represent the gap introduced to preserve sequence alignment;

Symbols "▼" indicates the two positions associated with virus phenotypes.

The initials of amino acids are listed in appendix I.

(a)NA96425

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consensus				CTRPNNNTRK	SIHIGPGRAF	YTTGEIIGDI	RQAHC					Clone	x	n	P
LN	AEEEVVIRSE	NFTNNAKTII	VQLNESVIIN	CIRPNNNTRK	RITMGPGRVY	YTTGEIIGDI	RKAHC	NISST	QWNKTLKGIV	RKLREQFNKT	IVFNQSSG	1/9	5	7	S
	....	....	....	....	....	....	.....	.....	........V.	K.........	....	1/9	5	7	S
	....I.....	......R...	.......V..	....	....	....	.....	.....	........V.	....	....	1/9	5	7	S
	....I.....	....	.......V..	.M........	....	....	.....	.....	........V.	....	....	1/9	5	7	S
	....I.....	....	.......V..	....	....	....	.....	.....	........A.	K.........	....	1/9	5	7	S
	....I.....	....	...S......	....	S.........	.....R....	.....	.....	L.........	K.........	....	1/9	5	7	N
	....	....	....	....	S.........	....D.....	.....	. ...	........V.	....	....	1/9	4	7	N
	....	....	....	....	S.........	....	.....	....	....	....	....	1/9	4	6	N
	....I.....	....	.....A.V..	.T........	S.........	....	.....	.....	..D.A.....	....	...T....	1/9	4	5	N
Lung	....I.....	....	.....P....	.T........	S.........	....	.....	.....	..D.A.....	....	...T....	2/8	4	5	N
	....I.....	..A.......	...T.P....	.T........	S.........	....	.....	.....	..D.A.....	....	...T....	1/8	4	5	N
	...GI.....	....	.....P....	.T........	S.........	....	.....	.....	..D.A.....	....	...T....	1/8	4	5	N
	....I.....	......R...	.....P....	.T........	S.........	....	.....	.....	..D.A.....	....	T..T....	1/8	4	5	N
	....I.....	....	.....P....	.T........	S.........	C.........	.....	.....	..D.A.....	....	...T....	1/8	4	6	N
	....I.....	....	.....P....	.T...S....	S.........	.I........	.....	.....	..D.A.....	....	...T....	1/8	4	7	N
	....I.....	....	.....P....	.T........	S.........	........G.	.....	.....	..D.A.....	....	...T....	1/8	5	6	N
Colon	....I.....	....	....	....	....	....	.....	.....	........V.	K.........	....	3/13	5	7	S
	....I.....	....	.......V..	....	....	....	.....	.....	........V.	K.........	....	3/13	5	7	S
	....I.....	.....T....	....	....	....	....	.....	.....	........V.	K.........	....	1/13	5	7	S
	....I.....	....S.....	....	....	....	....	.....	.....	........V.	K.........	....	1/13	5	7	S
	....	....	....	....	....	....	.....	S....	........V.	....	....	1/13	5	7	S
	....I.....	....	.......V..	...S......	....	....	.....	.....	........V.	K.....N	.....P..	1/13	5	8	S
	....I.....	....	....	....	....	..I.......	.....	.....	........V.	K.........	....	1/13	5	8	S
	....I.....	....	....	....	......D...	....	..T..	.....	........V.	K.........	....	1/13	4	9	S
	....I.....	....	....	.........E	...T......	....	.....	.....	........V.	K.........	....	1/13	3	8	S

(b)NA96371

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consensus				CTRPNNNTRK	SIHIGPGRAF	YTTGEIIGDI	RQAHC					Clone	x	n	P
LN	AEEEVVIRSD	NFSDNAKTII	VQLKESVEIN	CTRPNNNTRR	SIPIGPGRAF	YTTGDIIGDI	REAHC	NLSRA	KWTETLQQIV	IKLREQFGNK	TIVFNSSSG	3/12	2	4	N
	.....	.....	.....	......D...	.......K..	.A.....	.Q...	.....	..N.....	.....	.....	1/12	2	6	N
	.....	.....	.....	.....	.......K..	.A.....	.Q...	.....	..N..P....	.....	.....	1/12	3	5	N
	.....	.....	.....	.....	.....	.A.....	.Q...	....T	..N.....	.....R	.....	1/12	3	4	N
	.....	.....	.....	.....	.....	.A.....	.Q...	.....	..N.....	.....R	.....	2/12	3	4	N
	.....	.....T....	.....	........SK	RVTL...KVW	.....V...	.Q...	.....	..N.....	.....	.....	1/12	3	10	S
	.....	.....	.....	........SK	RVTL...KVW	.....V...	.Q...	.....	..N.....	.....	.....	2/12	3	10	S
	.....	.....	.....	.........K	RVTL....VW	.....MV...	.....	.....	..ND....V.	.....Y.	.....	1/12	3	10	S
Lung	.....	.....	.....	.....	.......G..	.....	.....	.....	..N.....	.....	.....	1/12	1	5	N
	.....	.....	.....	.....	...V.....	.....	.....	.....	..N.....	.....	.....	1/12	2	5	N
	.....	.....	.....	.........K	..N.....	.....	.....	.....	..N.....	.....	.....	3/12	2	3	N
	.....	.....	.....	.........K	..N.....	.....	.....	S....	..S.....V.	.....	.....	1/12	2	3	N
	.....	.....	.....	.....	.....	.....	.....	.....	R.N.....	.....	.....	1/12	2	4	N
	.....	.....	.....	.....	.....	.....	.....	.....	..N.....	.....	.....	1/12	2	4	N
	.....	.....	.....	.....	.......K..	.A.....	.....	.....	..N.....	.....	.....	1/12	2	6	N
	.....	...G.....	.....	.....	.......K..	.A.....	.Q...	.....	..N...K...	.....	.....	1/12	3	5	N
	.....	.....	.....	.........K	RVTL....VW	.....V...	.....	.....	..N.....	.....	.....	1/12	3	9	S
	.....	.....	.....	.........K	R.TL....VW	.....V...	.Q...	.....	..N.....	.....	.....	1/12	4	7	S
	.....	.....	.....	.........K	R.TL.....W	.....V...	.Q...	.....	..N.....V.	.......K..	....K....	1/12	4	6	S
LF	.....	.....	.....	........SK	RVTL...KVW	.....V...	.Q...	.....	..S..Q..V.	.....	.....	1/10	3	10	S
	.....	.....	.....	........SK	RVTL...KVW	.....V...	.Q...	.....	..S.....V.	.....	.....	1/10	3	10	S
	.....	.....	....G.....	........SK	RVTL....VW	.....VV...	.Q...	.....	R.S.....V.	.....	.....	1/10	3	10	S
	.....	.....	.....	.........K	RVTL....VW	.....V...	.Q...	S....	..N.....	.....	.....	2/10	4	8	S
	.....	V	.....	.........K	RVTL....VW	.....V...	.Q...	.....	..N.....	.....	.....	1/10	4	8	S
	.....	...G.....	.....	.........K	RVTL....VW	.....V...	.Q...	.....	..N.....	.....	.....	1/10	4	8	S
	.....	.....	.....	.........K	RVTL....VW	.....V...	.Q...	.....	..N..Q....	.....	.....	1/10	4	8	S
	.....	.....	.....	.........K	RVTL....VW	.....V...	.Q...	.....	..N.....	.....	.....	1/10	4	8	S
	.....	.....	.....	.........K	RVTL....VW	...V..V...	.Q...	.....	..S.....V.	.....	.....	1/10	4	9	S

(c)NA97020

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consensus		CTRPNNNTRK SIHIGPGRAF YTTGEIIGDI RQAHC		Clone	x	n	P
LN	AEEEVVIRSA NFTNNAKTII VQLNKSIEIN	CTRPSNNTSR GINIGPGRAF YTTERITGDI RQAHC	NISRA DWNKTLQQIV EKLREQFGNN KTIVFNQSSG	4/10	3	8	S
	....SD...N..	....K.....	....	1/10	3	8	S
	....SD...N..	..... S.H..... DA..K.....	.L..T A.....HH.. .....E..K. ...E.KG...	2/10	2	8	S
	....SD...N..	..... S.H..... .A..K.....	.L..T ......HH.. .....E..K. ...E.KR...	1/10	3	7	S
	....SD.....	..... S.H..... .A..K.....	.L..T A.....HH.. .....E..K. ...E.KR...	1/10	3	7	S
	....SD.....	....N..... R.A.....L F..DK.....	.L..T ......HH.. .....K..K. ...E.KR...	1/10	4	9	S
Lung	....	....	....	6/9	3	8	S
	....	....	....R....	1/9	3	8	S
	....	....K.....	....H...	1/9	3	8	S
	....S.....	.....G.	....	1/9	3	8	S
LF	....SD.....	....N..... R.R.....V IAADK.....	.L..T ......HH.. .....K..K. ...E.RR...	1/8	5	10	S
	....SD.....	....N..... R.R.....V IAADK.....	.L..T ......HH.. .E...K..K. ...E.KR...	1/8	5	10	S
	....SD.....	....N..... R.R.....V IAADK.....	.L..T ......HH.. .....N..K. ...E.KR...	1/8	5	10	S
	....SDY.....	....N..... R.R.....V IAADK.....	.L..T ......HH.. .....K..K. ...E.KR...	1/8	5	10	S
	....SD..... ...S.....	....N..... R.R.....V IAADK.....	.L..T ....A.HH.. .....K..K. ...E.KR...	1/8	5	10	S
	....SD.....	....N..... R.R.....V IAADK.....	.L..T ...R..HH.. .....K..K. ...E.KR...	1/8	5	10	S
	....SD.....	....N..... R.R.....V IAADK.....	.L.K. ......HHV. .....K..K. ...E.KR...	1/8	5	10	S
	....SD.....	....N..... R.R...R..V IAADK.....	.L..T ......HH.. .....K..K. ...E.KR...	1/8	6	10	S

(d)NA97017

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consensus				CTRPNNNTRK SIHIGPGRAF YTTGEIIGDI RQAHC				Clone	x	n	P
LN	AEEEVVIRSE NFTNNAKVII VQLNETVEIN			CTRPNNNTRK SITIGPGKAF YATGDIIGDI RQAHC		NISGA RWNNTLKQIV IKLREQFR-N KTIIFNQSSG		1/10	3	4	N
	.....S.....			....		....T ....-. .....S....		1/10	3	4	N
	....			..... G.....		....T ....-.		2/10	3	5	N
	....			....		....T ....-.		6/10	3	4	N
Lung	....I..... ..S....N.. ....KS.A..			.....S.... G.H.A..T.. .T.....		.L.K. E.E...R.VA .....Q-. ...V.....		1/11	2	6	N
	....I..... ..S....N.. ....KS.A..			..... G.H.A..T.. .T.....		.L.K. E.E...R.VA .....Q-. ...V.....		3/11	2	5	N
	....I..... ..S....N.. ....KS.A..			..... G.H.A..T.. .T.....		.L.K. E.E...G.VA .....Q-. ...V.....		1/11	2	5	N
	....I..... ..S....N.. ...TKS.A..			....H.A..T.. .T.....		.L.K. E.E.....VA .....E-. ...V.....		1/11	2	4	N
	....I..... ..S....N.. ...TKS.A..			.....G. ..H.A..T.. .T.....		.L.K. E.E.....VA .....E-. ...V.....		1/11	1	5	N
	...DI..... ..S....N.. ...TKS.A..			....H.A..T.. .T..G.....		.L.K. E.E...E.VA .....E-. ...V.....		1/11	3	4	N
	....I....S ...D...I.. ....KSIP.H			.I..... ..P....R.. ....E.....		.L.RT D..... .....K-. ...V.....		1/11	3	3	N
	....I....S ...D...I.. ....KSIP.H			.I..... ..P....R.. ....E.....		.L.RT D..... .....Q-. ...V.....		1/11	3	3	N
	....I....S ...D...I.. ....KSIP.H			.I..... ..PT...R.. ....E.....		.L.RT D..S..... .....K-. ...V.....		1/11	3	4	N
Colon	.....S .....I.. ....KS.P..			.I..... ..P....R.. .T..E.....		.L... E..... .....GK. ...V.....		6/8	3	2	N
	.....S .....I.. ....KS.P..			.I..... ..P....R.. .T..E.....		.L... E.D..... .....GK. ...V.....		1/8	3	2	N
	.....S .....I.. ....KS.P..			.I..... ..P....R.S .T..E.....		.L... E..... .....GK. ...V.....		1/8	3	3	N

(e)NA96272

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consensus		CTRPNNNTRK SIHIGPGRAF YTTGEIIGDI RQAHC		Clone	*x*	*n*	P
LN	AEEEVVIRSE NFTDNAKIII VQLNASVEIN	CTRPNNNTRK SIHIGPGRAF YTTGEIIGDI RQAHC	TLSRA KWNDTLKQIV TKLREQFKNK TIVFNQSSG	6/11	3	0	N
	....N.....	....	....	1/11	3	0	N
	....V .....F....	....	....	1/11	3	0	N
	....	..G.......	.... .R........	1/11	2	1	N
	....	....A...N.	NI..S ....A	1/11	5	2	N
	....N...V..	....N....... ....A...N.	NI..S ....A ....E.. ....SP...	1/11	5	3	N
Lung	....N...V..	....N....... ..A.A.....	N..G. ....A ....E.. ....SP...	1/10	4	3	N
	....N...V..	....N....... ....A.....	N.... ....A ....E.. ....SP...	1/10	4	2	N
	....A..... ...N...V..	....N....... ....A.....	N.... ....A ....E.. ....SP...	1/10	4	2	N
	....N...V..	....N....... ....A.....	N.... ....A ....E.. .T..SP...	2/10	4	2	N
	...G...... ...N...V..	...S...... ..N....... ....A.....	N.... ....A ....E.. .T..SP...	1/10	4	3	N
	....N...V..	....N....... ....A...N.	NI..S ....A ....E.. ....SP...	1/10	5	3	N
	....N...V..	....N....... ....A...N.	NI..S ....A ......SE.. ....SP...	1/10	5	3	N
	....N...V.. ......A...	.....H.... ..N....... ....N.	NI..S ....A ....E.. ....K....	1/10	4	3	N
	....N...VT. ......A...	.....H.... ..N....... ....N.	NI..S ......NK.A ....E.. ....K....	1/10	4	3	N
Colon	.........D	....	N.... .... I......G..	1/10	3	0	N
	.........D	....S....	N.... .... I......G..	1/10	3	1	N
	....N...V..	....N....... ....T.....	N.... .... I......G..	1/10	4	2	N
	....N...V..	....N....... ....A.....	N.... ....A ....E..	1/10	4	2	N
	....N...V..	....N....... ....A.....	N...S ....A ....E.. ....SP...	1/10	4	2	N
	....N...V.. ....E.....	....N....... ....N. ...Y.	NI..L ..EN...K.. ....E..	1/10	4	2	N
	....N...V..	....N.	NI..S ....A ....E.. ....SP...	1/10	4	1	N
	....N...V..	....N.	NI..S ...A.....A A......E.. ....SP...	1/10	4	1	N
	....N...V..	....N....... ....A...N.	NI..S ....A ....E.. ....SP...	1/10	5	3	N
	....N...V..	....N....... ....A...N. .P...	NI..S ....A ....E.. ....SP...	1/10	5	3	N

(f)NA97021

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consensus		CTRPNNNTRK SIHIGPGRAF YTTGEIIGDI RQAHC		Clone	x	n	P
LN	AEEEVVIRSE NFTNNAKVII VQLNETVEIN	CTRPNNNTRK SITMGPGKAF YATGDIIGDI RQAHC	NISGT KWNNTLKQIV IKLREQFG-N KTIIFNQSSG	2/10	3	5	N
	.....S...	....	..... R.....R... .....-. ..M.....	1/10	3	5	N
	....	....	..... R.....R... .....-.	2/10	3	5	N
	....	....	..... R..... .....-.	4/10	3	5	N
	....V	....	..... R..... .....-.	1/10	3	5	N
Lung	....	....	..... R..... .....-.	2/10	3	5	N
	....	.... D..... .R...	..... R..... .....-.	1/10	3	7	N
	....	....	....-. .I.....	1/10	3	5	N
	....	....P.....	...R. ...K..... .....-. ...A.....	1/10	3	5	N
	....	....P.....	...R. R..... .....-. ..M.....	1/10	3	5	N
	....	....	...R. R..... .....-. ..M.....	1/10	3	5	N
	....	....	...R. R..... .....-.	1/10	3	5	N
	....	....	...R. R.....R... .....-.	1/10	3	5	N
	....	....	...R. R..K...R.. .....-.	1/10	3	5	N
Colon	....P.K.....	....R.....	....R-.	1/10	4	6	N
	....K.....	....R.....	....R-.	1/10	4	6	N
	....I.....	....	..... R..... .....-. ..M.....	1/10	3	5	N
	....I.....	....PI.....	..... R..... .....-.	1/10	3	4	N
	....	....P.....	...RK Q..K.....A .....N. ...A..H...	1/10	3	5	N
	....N..	....P.....T.	...R. ...K.....A .....N. ...A..H...	2/10	3	6	N
	....N..	.....A.. ..P.....T.	...R. ...K.....A M.....N. ...A..H...	1/10	3	7	N
	....N..	....P.....T.	...R. ...K.....A M.....N. ...A..H...	1/10	3	6	N
	....N..	....P.....T.	...R. ...K..... .....N. ...A.....	1/10	3	6	N
LF	....A....N..	....P.....	...R. ...K..... .....N.	1/10	3	5	N
	....N..	....P.....	...R. ...K..... .....N.	4/10	3	5	N
	....N..	....P..... ..... .R...	...R. ...K..... .....N.	1/10	4	6	N
	....N..	....P..... .....N.	...R. R..... .....-.	2/10	4	6	N
	....N..	....P..... .....N.	...R. R.....L. .....-.	1/10	4	6	N
	....N..	....P..... .....N.	...R. R..... .....-. .....P..	1/10	4	6	N

(g)NA98025

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consensus		CTRPNNNTRK SIHIGPGRAF YTTGEIIGDI RQAHC		Clone	x	n	P
LN	AEEEVVIRSQ NFTNNAKVII VQLNESVVIN	CTRPNNNTRK SIHIGPGRAF YTTGEITGDI RKAHC	NVSRA KWENTLKQIA LKLGEQFG-N KTIVFNQSSG	2/9	4	2	N
	.....A..	....	....-.	1/9	4	2	N
	.....F.	....	....-.	1/9	4	2	N
	....	....QR.....	....D..... .....-.	1/9	5	4	S
	....	....Q.I... ...Y.	E.N.. E.K..... .....-.	1/9	5	4	S
	....	....Q.I... .Q.Y.	E.N.. E.K..... .....-.	1/9	4	3	N
	....D..... ....K.....	....Q.I... .Q.Y.	..... E.K.....V P..R....N.	1/9	4	3	N
	....	.A..... ....Q.I... .Q.Y.	..... E.K.....V P..RG...N.	1/9	4	3	N
Lung	....D	....I.PM .Q.Y.	SL... ..ND.....V I..R...K-S	1/10	4	3	N
	...G..... ....D	....I.P. .Q.Y.	SL... ..ND.....V I..R...K-S	1/10	4	2	N
	....D	....I.P. .Q.Y.	SL... ..ND.....V I..R...K-S	1/10	4	2	N
	....A..... .....D	....I.P. .Q.Y.	SL... ..ND.....V I..R...K-S	1/10	4	2	N
	....D	....I.P. .Q.Y.	SL... ..ND.....V ...K....-. .I.....	1/10	4	2	N
	....D	....I.P. .Q.Y.	SL... ..N.....V ...K....-. .I.....	2/10	4	2	N
	.....K ...D.....	....I.P. .Q.Y.	E.... G.N.....V ...K....-.	1/10	4	2	N
	.....K ...D.....	....I.P. .Q.Y.	E.... E.N.....V ...K....-.	1/10	4	2	N
	.....I...K ...D.....	....I.P. .Q.Y.	E.... E.N.....V ...K....-.	1/10	4	2	N
Colon	....D	....I.PT .Q.Y.	SL... ..ND.....V I..R...K-S	1/10	4	3	N
	....D	....I.P. .Q.Y.	SL... ..ND.....V I..R...K-S	3/10	4	2	N
	....D..... .....D	....I.P. .Q.Y.	SL... ..ND.....V I..R...K-S	1/10	4	2	N
	....D.....	....I.P. .Q.Y.	SL... ..ND.....V I..R...K-S	2/10	4	2	N
	.....K ...D.....	....I.N. .Q.Y.	E.... E.N.....V ...K....-.	1/10	4	2	N
	....	....I.Q. .Q.Y.	E...V E.N.....V ...K....-.	1/10	5	2	N
	....D	..G..... ....I... .Q.Y.	..... E.K.....V P..R....N.	1/10	2	1	N
LF	.....K	.... .A....I.EV .Q.Y.	E.... E.N.....V ...K.H..-.	1/10	3	4	N
	.....K	.... .A....I.E. .Q.Y.	E.... E.N.....V ...R....-.	1/10	3	3	N
	.....K	.... .A....I.E. .Q.Y.	E.... E.N.....V ...K....-.	2/10	3	3	N
	.....K	.....D.... .T..... .A....I.E. .Q.Y.	E.... E.N.....V ...K....-.	1/10	2	4	N
	.....K ...D.....	.I...D.... ....E..I.E. .Q.Y.	SL... ..S.....V I..R...K-. .....K....	1/10	1	5	N
	.....K ...D.....	.I..... ....E..I.E. .Q.Y.	SL... ..S.....V I..R...K-. .....K....	3/10	2	4	N
	.....K ...D.....	....M..... .....I.Q. .Q.Y.	E.... E.N.....V ...R....-. ..R.....	1/10	4	3	N

(h)NA98028

271 ▼ ▼ 369

consensus		CTRPNNNTRK SIHIGPGRAF YTTGEIIGDI RQAHC		Clone	*x*	*n*	P
LN	AEEEVVIRSD NFTDNAKIII VQLNESVEIN	CTRPNNNTRK SIHIGPGRAF YTTGEIIGDI RQAHC	NLSRA KWDDTLKQIV IKLREQFENK TIVFNQSSG	7/9	3	0	N
	....V..	....	....T E..A...R.. .....K.... ..A.K....	1/9	3	0	N
	....	....	....R..	1/9	3	0	N
Lung	....	....	....K..	2/11	3	0	N
	....V..	....	....K.. ..... .T.....	1/11	3	0	N
	....	....	....EY...K..	1/11	3	0	N
	....	....	....T E.....K.. .....K.... ..A.K....	1/11	3	0	N
	....V..	....	....N...K.. .....K.... ..A.K....	1/11	3	0	N
	....V..	....	....T E..V...K.. .....K.... ..A.K....	1/11	3	0	N
	....V..	....	....T E..A...T.. .....K.... ..A.K....	1/11	3	0	N
	....V..	....	....T ...A...K.. ....A.K....	1/11	3	0	N
	.....FG.. D.....	....	....N...K.. ....A.K....	1/11	3	0	N

(i)NA97097

271 ▼ ▼ 369

consensus		CTRPNNNTRK SIHIGPGRAF YTTGEIIGDI RQAHC		Clone	x	n	P
LN	AEEEVVIRSE NFTNNAKVII VQLNESVAIN	CTRPGNNTRR GIHIGPGSAF YTTGQIIGDI RQAYC	NISRK DWNTTLEKIV IKLREQFGNK TIVFNQSSG	4/10	3	6	N
	....	....V..	....	1/10	3	7	N
	....	....V..	....N..K...	1/10	3	7	N
	....	....R..	....E ...N..K..A ...GD..R.. ..A......	1/10	4	5	N
	....	....N...IK ....R..	....E ...N..K..A ...GD..R.. ..A......	1/10	3	4	N
	....	....N...IK ....R..	....	1/10	3	4	N
	....	..G.....	....	1/10	2	7	N
Lung	....	....	....	6/9	3	6	N
	....	.........K	....	1/9	3	5	N
	....	....L	....	1/9	3	7	N
	....	....	....K... .E........	1/9	3	6	N
LF	....K..E.K	.I..S..... ....E..... ...H.	.L... ..HD..K..A ...GD..... ..A......	2/8	2	5	N
	....K..E.K	.I..S..... ....E..... ...H.	.L... G.HD..K..A ...GD..... ..A......	1/8	2	5	N
	....K..E.K	.I..S..... ....L ....E..... ...H.	.L... ..HD..K..A ...GD..... ..A......	1/8	2	6	N
	....K..E.K	.I..S..... ....L ....E..... ..VH.	.L... ..HD..K..A ...GD..... ..A......	1/8	2	7	N
	.... D......... ....K..E.K	.I..S..... ....E..... ...H.	.L... ..HD..K..A ...GD..... ..A......	1/8	2	5	N
	....E... ....K..E.K	.I..S..... ....E..... ...L.	.L... ..HD..K..A ...GD..... ..A......	1/8	2	5	N
	....K..E.K	.I..S..... ....E..... ...H.	.L... .RHD..K..A ...GD..... ..A......	1/8	2	5	N

5.3.3 Partitioning of HIV-1 Variants in Different Tissues

Phylogenetic analysis was carried out using sequences from the V3 and $p17^{gag}$ regions from a range of lymphoid and non-lymphoid tissues, including LN, lung, colon and LF brain tissue. Divergence between nucleotide sequences was estimated using Jukes-Cantor distances (scale indicated below tree), and the phylogenetic tree constructed from the distance matrix by the neighbor-joining method. The robustness of groupings was indicated by bootstrap resampling of 100 datasets, with values of ≥70% indicated on branches. The tree was rooted using the sequence of HXB2 as an outgroup.

Phylogenetic analysis of nucleotide sequences for the V3 and $p17^{gag}$ regions revealed a variety of relationships between variants recovered from different tissues. In general, most of the HIV-1 variants amplified in both the V3 and $p17^{gag}$ regions from lung were interspersed with those from LN (Figure 5. 3). However, in subject NA98025, the majority of lung variants were interspersed with those from colon but separated from LN variants in the V3 region. Separate groupings of lung variants were observed within subjects NA96425, NA96371 and NA96272, although in subject NA96371, lung-specific lineage was only detected in the V3 region. There was evidence of a lung-specific lineage in subject NA96425, in whom all of the lung variants clustered separated from other variants isolated from colon and LN in the V3 region. Although a relatively restricted sequence diversity was observed in the V3 region (0.0093), the high level of proviral load measured in this lung sample indicates this homogeneous lineage did not result from restricted template copying.

The viral quasispecies from brain tissue were relatively homogeneous and commonly clustered separately from all other sequences, but occasionally with some colon and lung variants interspersed. For example, in subject NA97021, $p17^{gag}$ sequences of brain variants were extremely diverse and polyphyletic, but could be roughly divided into two clades. One clade contained most of the brain variants, all of the variants from LN and lung and most of the colon variants. The other clade was constructed with a minority of brain and colon variants. As for the V3 sequences, brain isolates were clustered into two distinct clades, both of which shared sequences

similar with the majority of colon variants, but separated from variants isolated from LN or lung. Also in subject NA98025, a separate grouping of brain isolates was observed in $p17^{gag}$ and V3 regions. Similar to the findings in subject NA97021, $p17^{gag}$ sequences of brain variants from subject NA98025 were diverse and polyphyletic. The majority of brain isolates clustered separately with variants isolated from LN and lung, but closely associated with the colon-specific lineage. Two distinct brain clades were identified in the V3 region, both of which were closely associated with two colon-lung lineages independently of each other (figure 5.3).

Sequence relationships between variants in the colon and those in other tissues were more complex. Evidence of a colon-specific lineage was observed within individuals NA97021, NA96272, NA96425 and NA98025, in whom part or all of the colon variants clustered separately from those isolated from lung or LN. In the latter two study subjects, a separate grouping was detected only in the $p17^{gag}$ region. In addition to those separately grouped, the remaining colon isolates were occasionally interspersed with those from lung (NA96272, V3; NA98025, V3), from LN (NA96425, V3), or interspersed with variants from LN and lung (NA96272, $p17^{gag}$; NA97021, V3).

Briefly summarising, variants recovered from LN, lung and colon are often interspersed. In contrast, variants recovered from brain are relatively homogeneous and clustered separately from those recovered from LN, but occasionally sharing sequences similar to colon and lung variants. Tissue specific groups were observed, most often within V3 region, although some of these occurred in the $p17^{gag}$ region, for example the colon-specific lineage in subject NA96425.

Figure 5. 3. Phylogenetic analysis of the nucleotide sequences from the V3 and p17^{gag} regions of (I) NA96425, (II) NA96271, (III) NANA97020, (IV) NA96272, (V) NA97021, (VI) NA98025, (VII) NA98028, (VIII)NA97097. Trees shown in rooted form using the sequence of HXB2 as an outgroup. Bootstrap values of ≥70% indicated on branches and highlighted in bold.

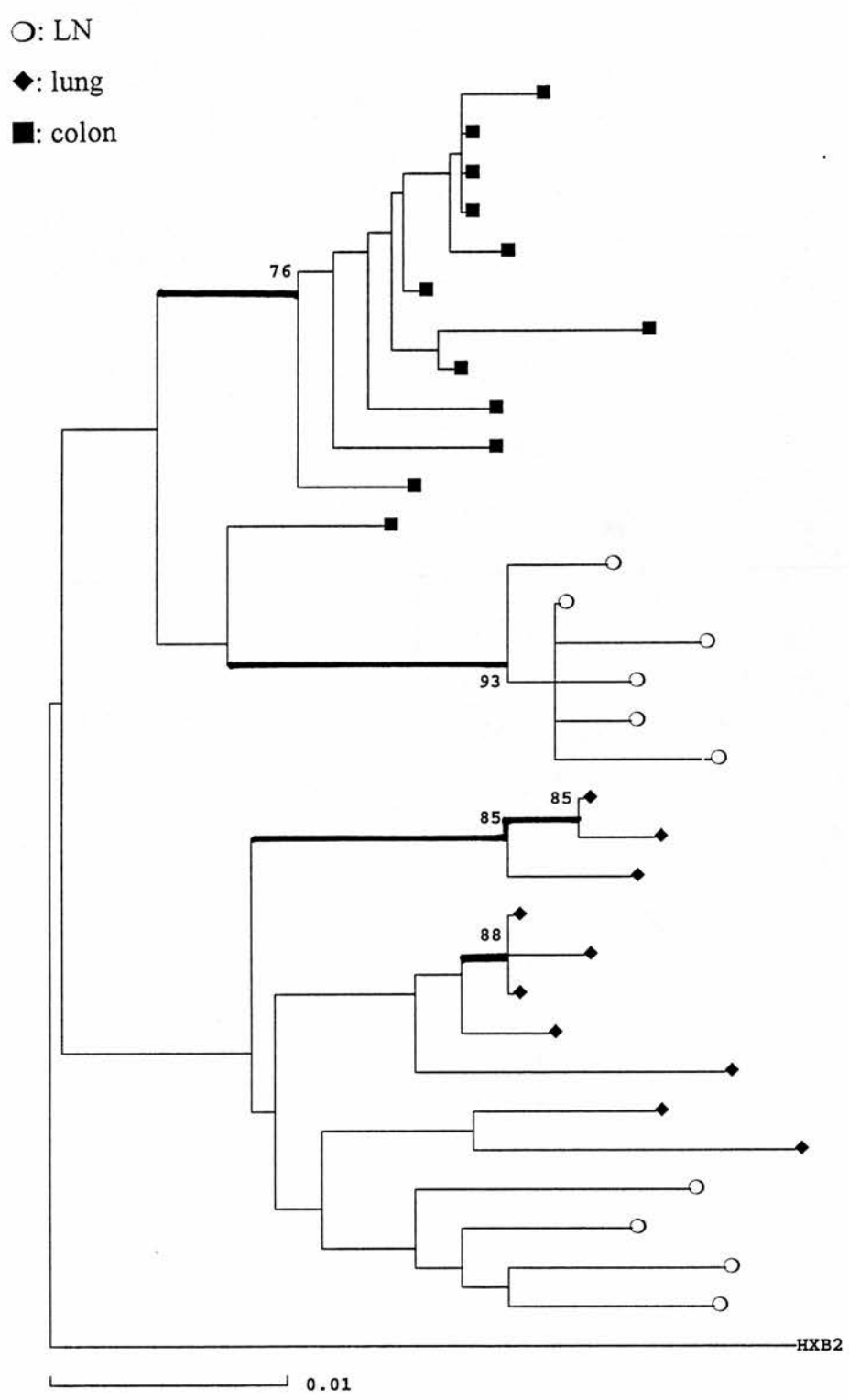
Symbols: ❍ indicates LN isolates

■ indicates colon isolates

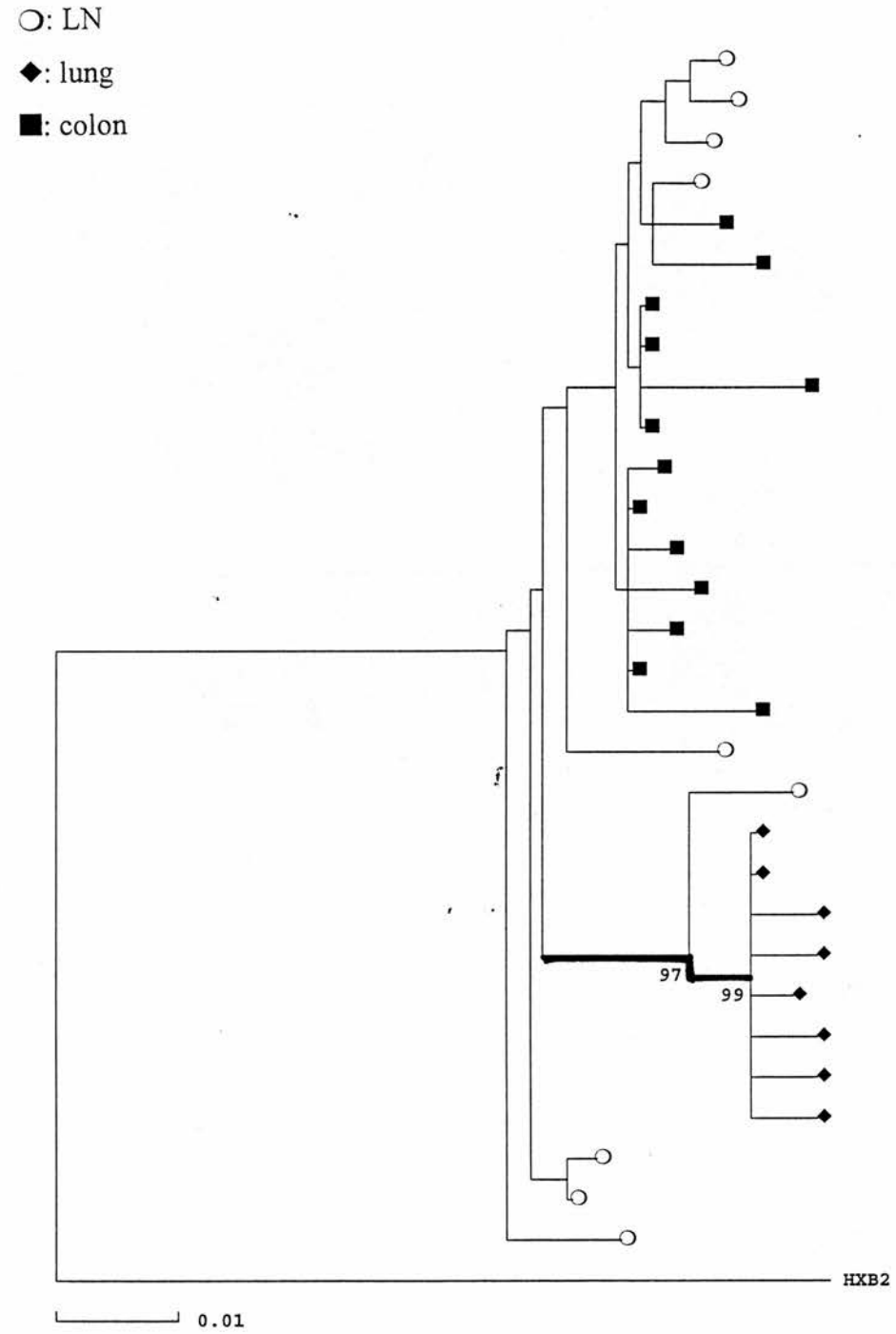
▲ indicates left frontal brain isolates

◆ indicates lung isolates

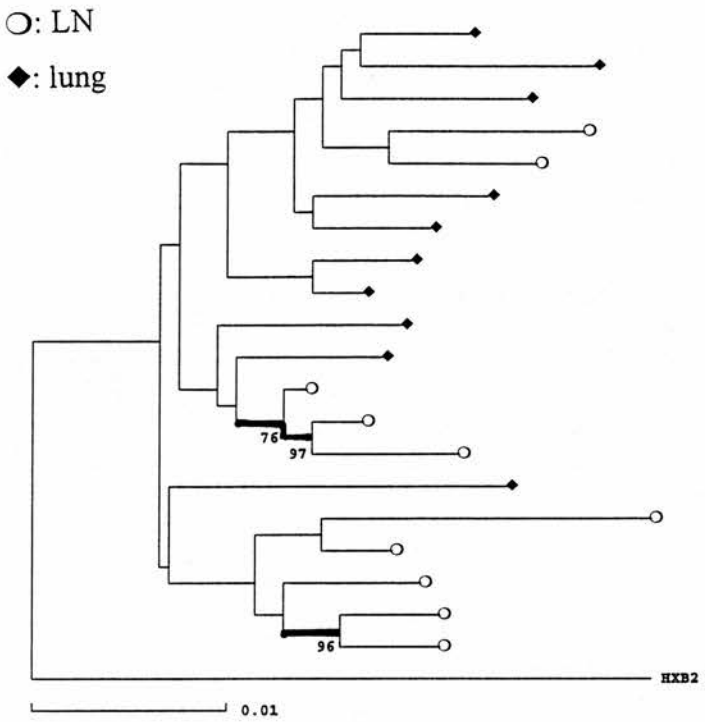
NA96425 Gag-p17



NA96425 Env-V3



NA96371 Gag-p17

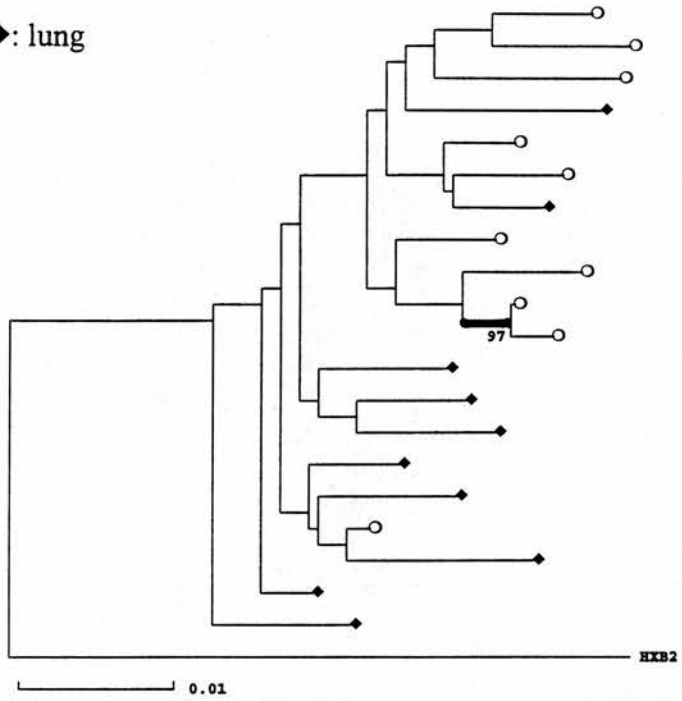


NA96371 Env-V3

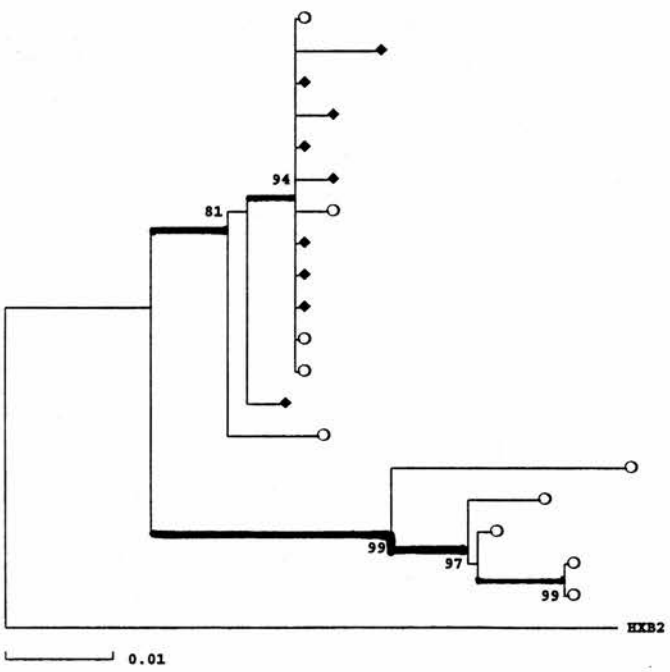
NA97020 Gag-p17

○: LN

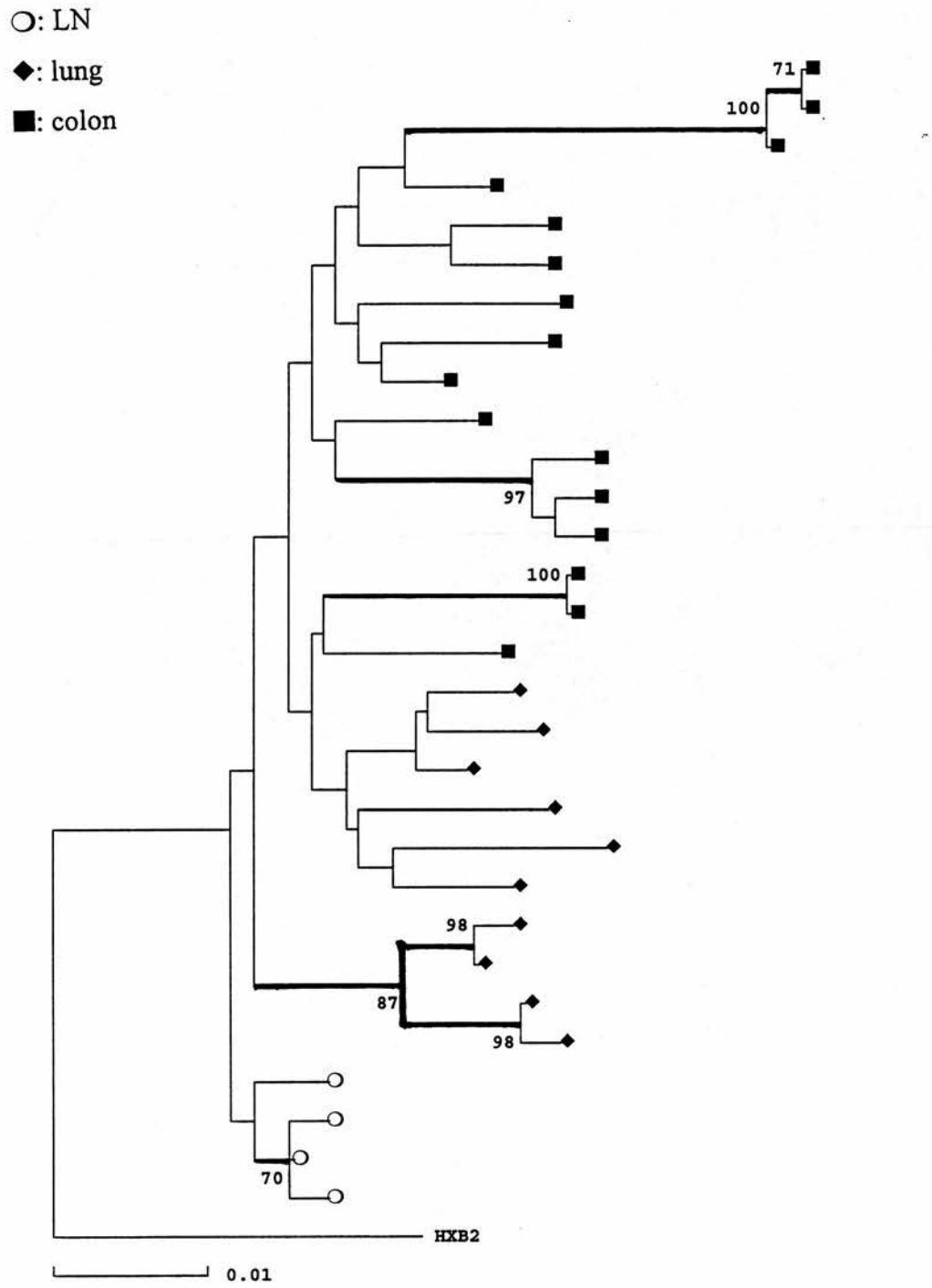
◆: lung



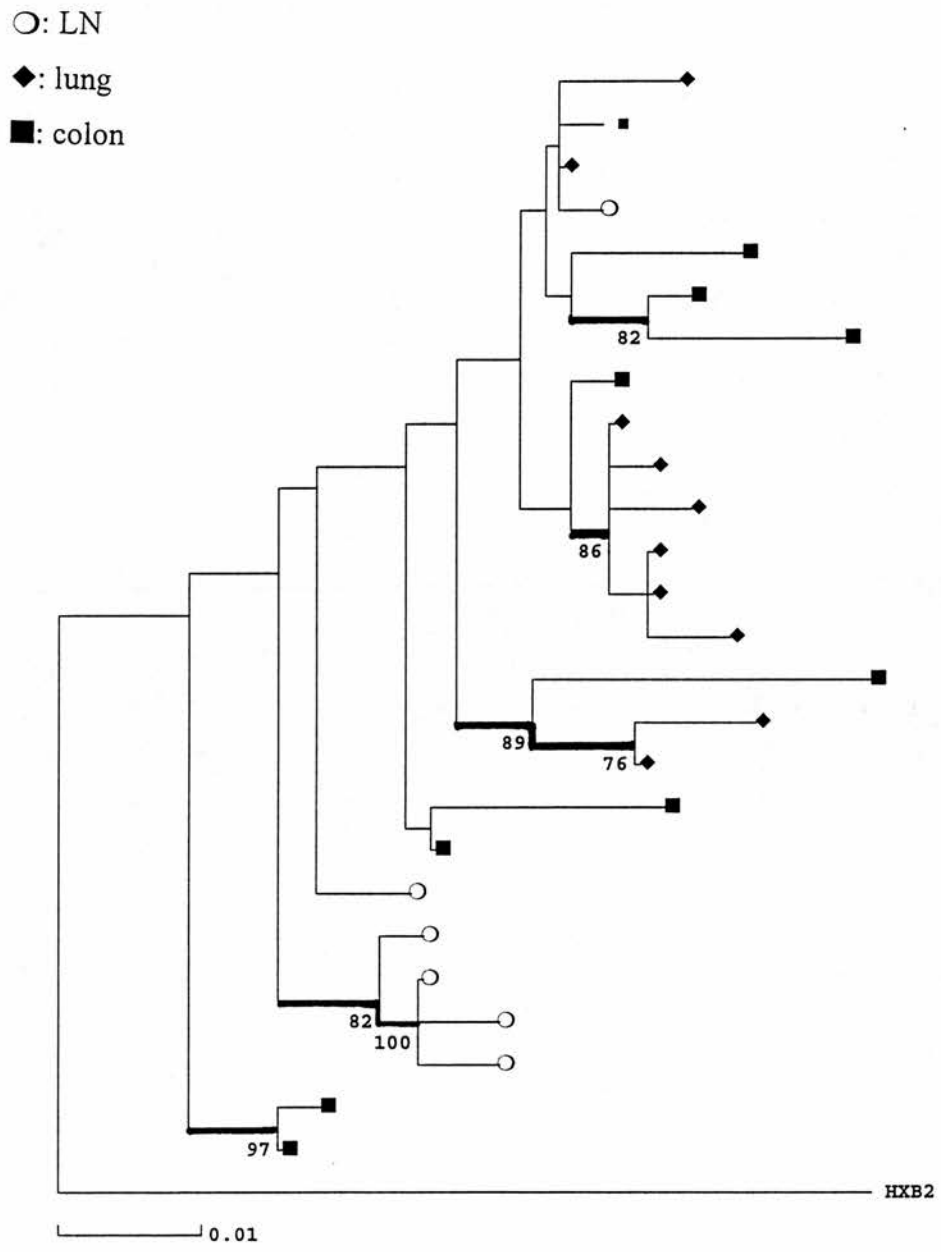
NA97020 Env-V3



NA96272 Gag-p17



NA96272 Env-V3



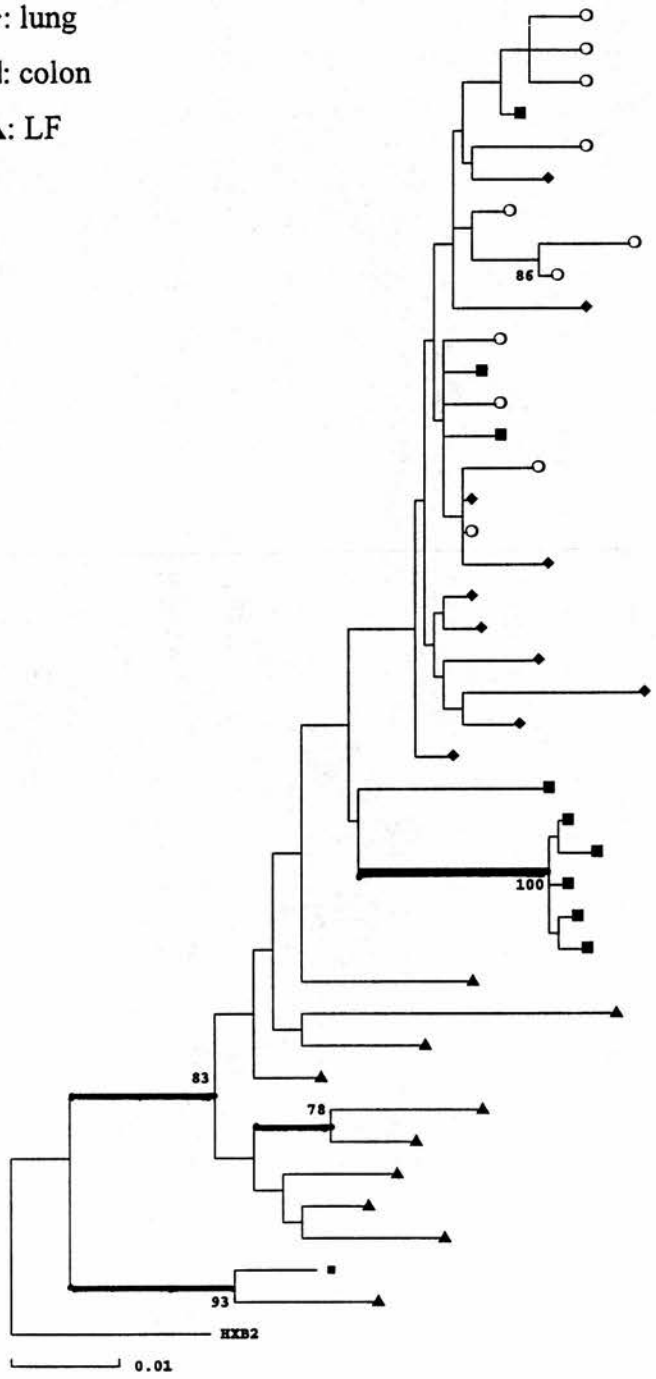
NA97021 Gag-p17

○: LN

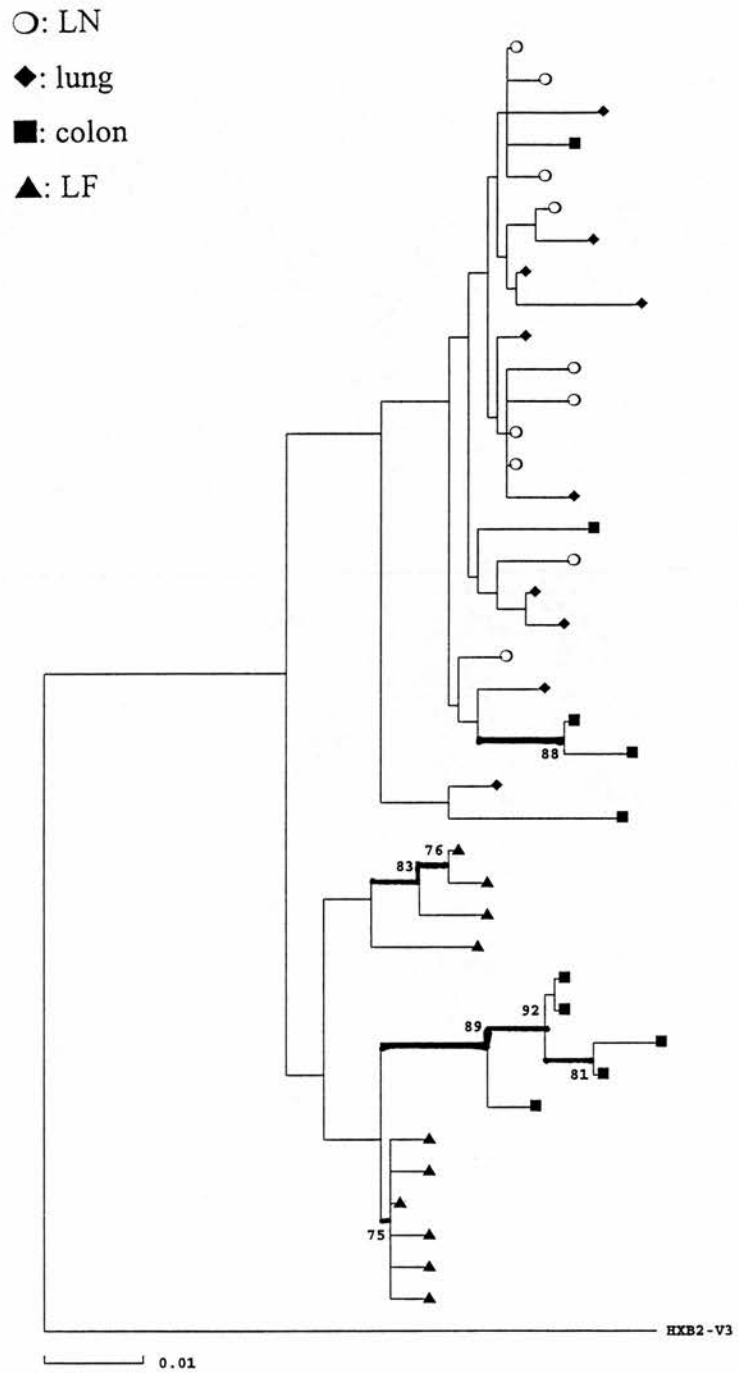
◆: lung

■: colon

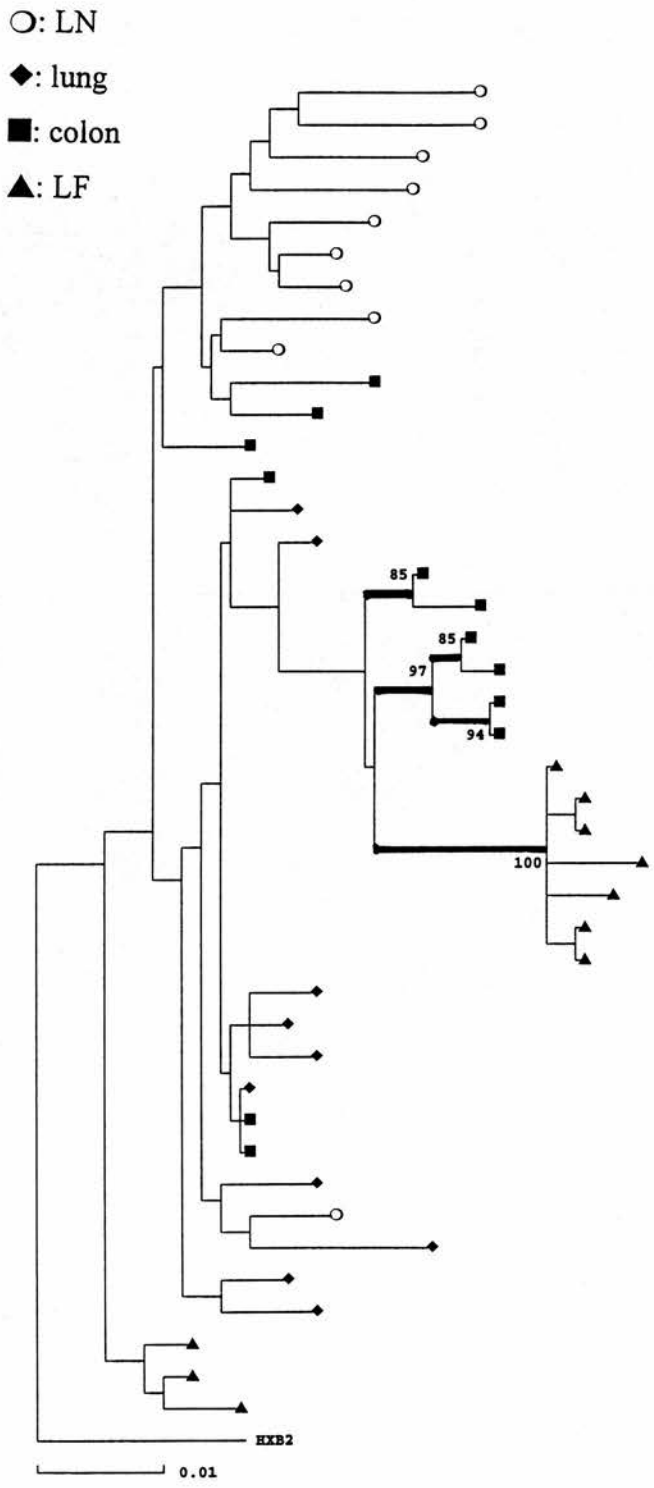
▲: LF



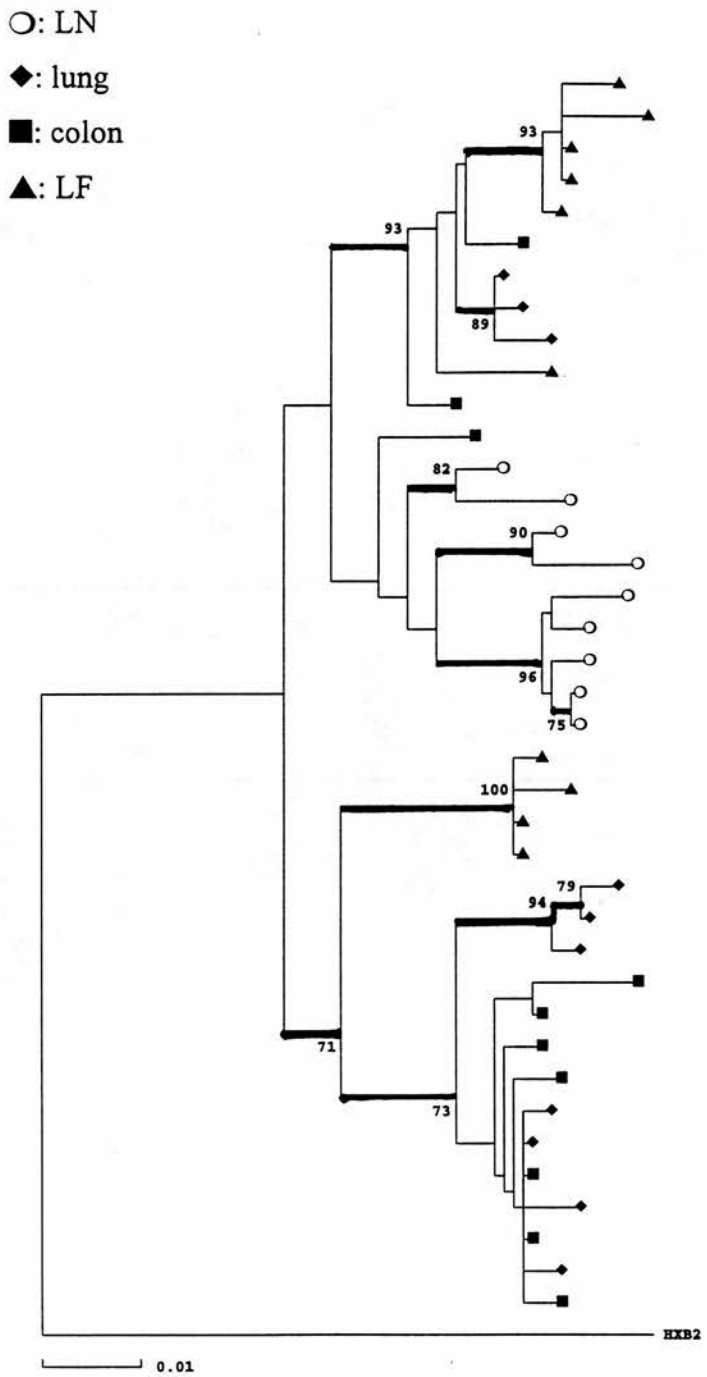
NA97021 Env-V3



NA98025 Gag-p17



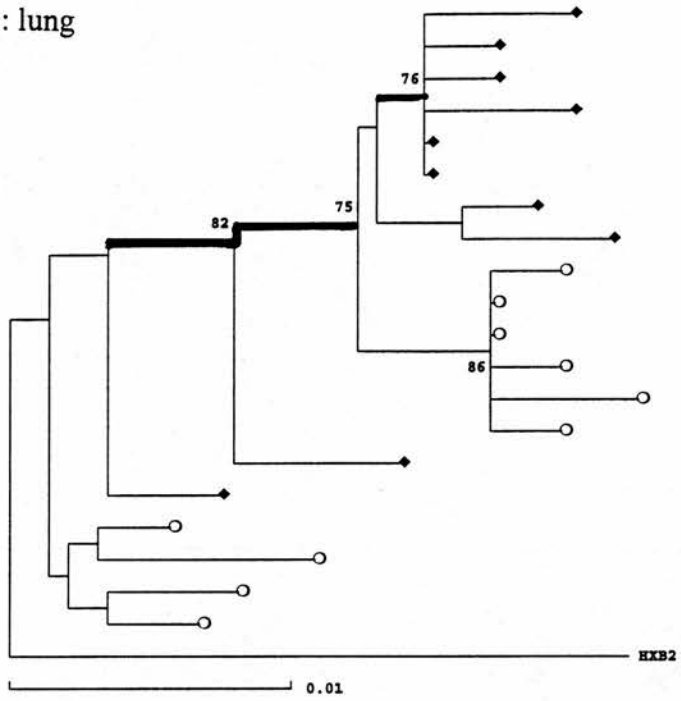
NA98025 Env-V3



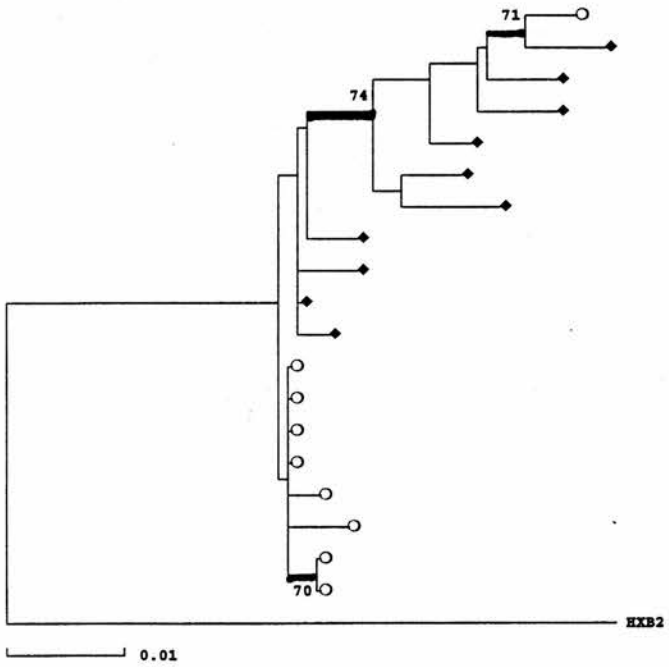
NA98028 Gag-p17

○: LN

◆: lung



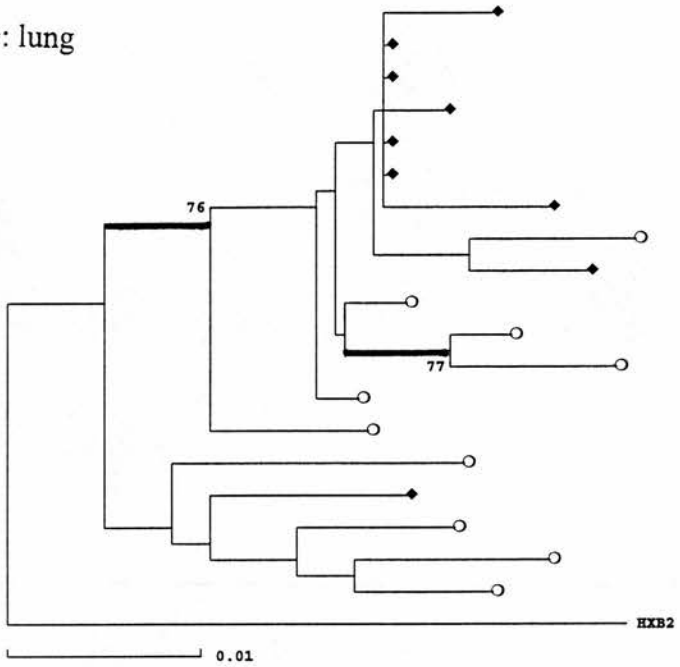
NA98028 Env-V3



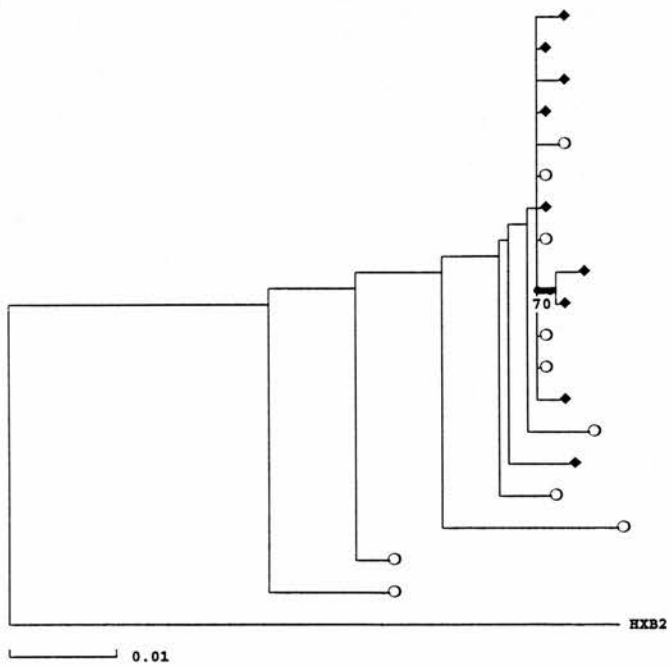
NA97097 Gag-p17

○: LN

◆: lung



NA97097 Env-V3



5.4 Discussion

5.4.1 Validity of the Nucleotide Sequence Results for Phylogenetic Analysis

It has been reported that the most effective thermostable DNA polymerase, *Taq* enzyme is often associated with a high misincorporation rate during nucleotide synthesis (Saiki *et al.,* 1988; Ennis *et al.,* 1990). Investigators have found that if the PCR product is cloned, the inserted DNA will be derived from sequences that may have been copied several times during the PCR, and such cumulative effects in sequence analysis could interfere with phylogenetic or evolutionary analysis (Leigh Brown & Simmonds, 1995).

The problem of single templates was highlighted by the observations of low sequence diversity (within the range of *Taq* error) together with low virus load within eight samples examined in this study. Cloned sequences of these samples were homogeneous, and produced a misleading indication of tissue-specific lineage. As described at section 5.3.1, the estimate for virus load in the brain sample from NA97097 was calculated from the frequency of 4 positive reactions from testing 10 replicate one μg aliquots of DNA. While this PCR product was cloned, it was likely that inserted DNA was derived from a single proviral template, and this resulted in a brain-specific lineage which actually represented only one molecule of proviral DNA from the tissue. Also in subjects NA96371 and NA97020, CXCR4-dependent variants were recovered from LF brain tissues, and these apparently grouped together as a brain-specific lineage. This is an unusual finding within isolates from brain tissue, since most of the published brain sequences are CCR5-dependent (reviewed in Ghorpade *et al.,* 1998). However, low viral load and little heterogeneity was observed within these two samples, and the amino acid sequences in the V3 region of these brain SI isolates demonstrated certain similarity with those SI strains recovered from LN and lung within these individuals (Figure 5. 2). It is difficult to determine whether the sequences obtained from samples with low virus loads are derived from the tissue itself or from residual blood within the tissue (Donaldson *et al.,* 1994a). Shared similarity in V3 sequences with variants recovered from other tissues suggested blood contamination as an origin for these SI brain variants. The

apparent brain lineage might only reflect one of the lymphoid variants (Figure 5. 4).

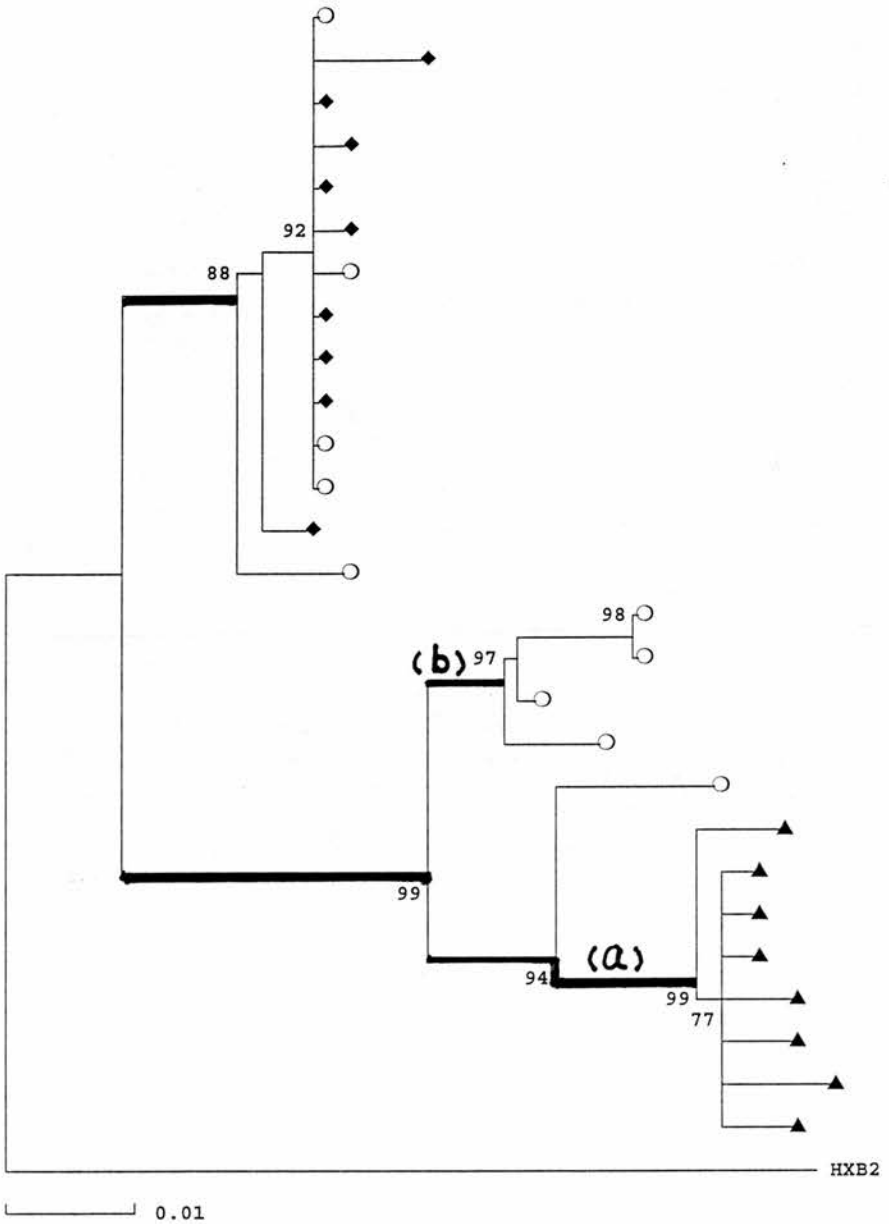


Figure 5. 4. Phylogenetic analysis of the V3 nucleotide sequences of individual NA97020. ❍ indicates LN isolates; ◆ indicates lung isolates; ▲ indicates left frontal brain isolates. The sequences involved in the observed brain-specific lineage (a) were relatively homogeneous and similar to those from LN in lineage (b). Due to the extremely low viral load in brain tissue (6 copies per 10^6 cells), and the similarity with LN isolates in branch b, this brain lineage (a) is highly likely to represent one of the lymphoid variants.

In addition, in patient NA97017, well-defined tissue specific grouping was observed in lung, LN and colon (Fig. 5.4). However, the presence of low viral load (approximately 10 copies per 10^6 cells) and less heterogeneity within variants isolated from colon and LN suggested that these variants might originate from a single template. The presence of extremely diverse lung sequences (0.0504 in V3) and (0.0137 in $p17^{gag}$) suggested that this high degree of heterogeneity could not result from *Taq* error. However as the proviral load was low (10 copies per 10^6 cells), these variants may have been amplified from residual blood within lung tissue and represent the general infection in circulation. Thus, in this case, the apparent tissue tropism was meaningless (Figure 5. 5).

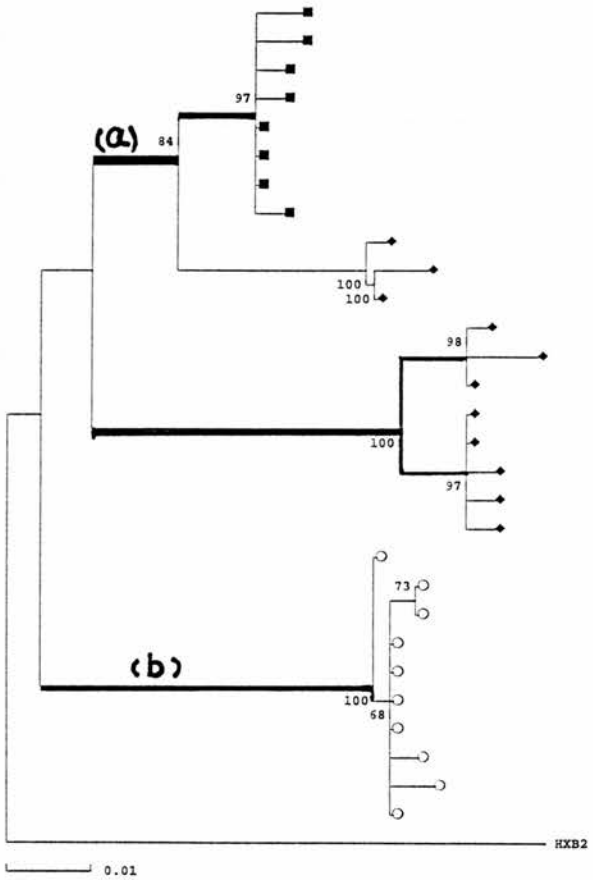


Figure 5. 5. Phylogenetic analysis of the V3 nucleotide sequences of individual NA97017. ❍ indicates LN isolates; ◆ indicates lung isolates; ■ indicates colon isolates. The problem of single template produces a mis-leading indication of tissue-specific lineages. As discussed above, lineage (a) and (d) were possibly derived from single proviral template.

To overcome the problem raised from the single template, limiting dilution PCR followed directly by cycle sequencing could be employed instead of cloning the sequence. Using limiting dilution PCR, a single molecule from the original sample pool can be amplified sufficiently for direct sequencing. The *Taq* errors could therefore be prevented, although the problem of contamination with residual blood is still difficult to solve.

5.4.2 Proviral Load, Sequence Relationships and Disease Progression

It is important to determine the dynamics of HIV-1 spread throughout the body in order to understand the mechanism of disease progression. Previous studies have detected HIV-1 proviral DNA in both lymphoid and non-lymphoid organs in patients who died in the symptomatic stages, but for those who died before the onset of AIDS, proviral DNA could only be detected within lymphoid organs (Donaldson *et al.*, 1994a). These findings provided evidence that spread of HIV outside cells of the lymphoid system is a late event in HIV infection (Donaldson *et al.*, 1994a; van't Wout *et al.*, 1998a). However, recent studies in evolutionary analysis of sequences from the $p17^{gag}$ region have demonstrated great sequence diversity in the brain, which showed several years of divergent evolution (Hughes *et al.*, 1997; Morris *et al.*, 1999), and this provided evidence of early entry of HIV-1 into CNS.

In this study, a highly sensitive, specific and accurate quantitative nested PCR was used for the detection of HIV-1 infection in various organs. Proviral DNA was persistently detected in LN and lung samples obtained from study subjects including both symptomatic and asymptomatic individuals, but was present in brain and colon only in symptomatic individuals. These findings, in agreement with previous studies (Donaldson *et al.*, 1994a; Reddy *et al.*, 1996) suggest that the detection of proviral DNA in non-lymphoid organs is sensitive to the degree of immunosupression in the patients. However, no evidence was observed for early entry of HIV-1 into CNS or colon in this group of patients.

As lymphoid organs have long been recognised as major virus reservoirs (Embretson *et al.*, 1993a; Pantaleo *et al.*, 1993c; Pantaleo *et al.*, 1997), it is not

surprising that proviral DNA was recovered from all lymph nodes in both presymptomatic and symptomatic stages. Most of the variants isolated from LN were predicted as CCR5-dependent/NSI strains, however there were some CXCR4-dependent/SI strains identified in those study subjects in a severely immunosuppressed condition. It has been reported that virus isolated in the early asymptomatic phase of infection is predominantly slowly replicating, macrophage tropic/NSI strains (Schuitemaker *et al.,* 1992; Zhu *et al.,* 1993; Blaak *et al.,* 1998). During disease progression, rapidly replicating, T-cell tropic viruses appear in about 50% of infected persons and are associated with the emergence of SI variants (Cheng-Mayer *et al.,* 1988; Tersmette *et al.,* 1989b; Schuitemaker *et al.,* 1992; Koot *et al.,* 1993). Some investigators suggested these SI strains might evolve from NSI strains (Tamalet *et al.,*1994; van't Wout *et al.,* 1998b). It is still unclear in which compartment the generation of SI variants occurs, since SI isolates recovered in this study are coincidentally associated with extremely low CD4 counts at death, suggesting that the emergence of SI strains might be accompanied by high levels of cytotoxicity *in vivo*, and then speed up the progression of disease to death.

In addition to the detection of HIV-1 in lymph node, HIV-1 proviral sequences were also detected at considerable levels in the lung tissues. Lung is an organ which is in particularly close contact with blood and is also known as a mucosa-associated lymphoid organ. Pulmonary complications are common in patients infected with HIV-1 and the correlation between the detection of HIV-1 proviral DNA in lung cells and progression to death has been observed in previous studies (Clarke *et al.,* 1995). In the present study, proviral DNA was consistently detected in the lung of all study subjects, including all three presymptomatic subjects, even in subject NA98028 who was without apparent abnormality observed in lung and still in the clinically latent stage at death. This observation, in agreement with Clark *et al.,* suggested the possibility of an early entry of HIV-1 into the lung (Clarke *et al.,* 1995). Most of the variants isolated from lung were interspersed with those from LN, but some were demonstrated unique lung lineages (NA96425) or interspersed with colon and brain isolates (NA98025). According to previous findings, where HIV-1 was present in both alveolar macrophages and alveolar lymphocytes (Clarke *et al.,* 1995; Agostini

et al., 1995; Nakata *et al.,* 1995; Semenzato *et al.,* 1995; Agostini *et al.,* 1996; Semenzato *et al.,* 1996), the different phylogenetic associations observed in this study might represent the different cell subtypes infected by HIV-1. CXCR4-dependent strains were identified in two study subjects, who had low CD4 counts at death, but were not present in the patient with the lowest CD4 count (NA96425). These SI strains were similar in sequences and phylogenetically interspersed with LN SI variants within the two individuals. Whether this represents a systematic viral re-invasion during the late stage of disease, or resulted simply because of the NSI/SI switch event happening at the end stage of disease, or was induced by other opportunistic infections needs further investigation.

HIV-1 infection in the gastrointestinal system has recently received more attention. Some investigators have reported that isolates recovered from the bowel can differ in biological and serological properties from HIV-1 strains recovered from serum (Barnett et al., 1991; van der Hoek et al., 1996). It has been reported that in homosexuals and infants, the gastrointestinal mucosa is the major route by which HIV-1 first enters host tissues (Smith et al., 1997). However, the present study failed to detect HIV-1 in the colon in the two male homosexuals in the symptomatic stage. The colon variants recovered in this study demonstrated a high level of heterogeneity and a complicated relationship with variants from other tissues. In general, a portion of colon variants appeared to be interspersed with LN isolates within individuals, and others were similar in sequences with variants recovered from the brain (NA97021; NA98025). Also, there was evidence of colon groups observed in the p17gag region (NA96425 and NA98025), and in the V3 (NA97021) region. Interestingly, all of the colon isolates from subject NA96425 were identified as CXCR4-dependent strains. These V3 sequences from colon were interspersed with those from LN in NA96425, who had been reported to have an extremely low CD4 count at death (CD4: 0), providing evidence suggesting that a systemic dissemination of infection occurred at the final stage. In a previous study, van der Hoek et al., also demonstrated SI strains in two of their study group (van der Hoek et al., 1998). These two individuals had been reported as having AIDS-related illness, however no other information was given on whether they were in the middle or late stage of symptomatic illness. How

these SI strains married to disease progression in this reported study is difficult to predict.

HIV-1 infection in the brain is known to be closely associated with advanced disease, particularly with HIV-encephalitis (HIVE), a neuroabnormality frequently found in AIDS individuals (Bell, 1998; Gatanaga *et al.,* 1999). Though HIV-1 proviral DNA was readily detected in some asymptomatic patients using the PCR technique (Sinclair *et al.,* 1992; Sinclair *et al.,* 1994; An *et al.,* 1996), the virus load was generally observed to be extremely low. In this study, the association between HIV-1 detection and HIVE was observed in subjects NA97021 and NA98025. The patient NA98025 was clinically still in the presymptomatic stage. However, the presence of occasional giant cells in the brain tissue suggested that this HIV positive man was developing incipient HIVE. This and the other subject, NA97021 who developed AIDS and HIVE, demonstrated similar sequence relationship between different tissues. Both of them presented a general lineage containing all LN variants, most lung variants, a small proportion of colon variants and few brain variants, together with unique brain and colon lineages. Moreover, the brain grouping demonstrated a close association with colon variants, in one case included some lung variants (NA98025). These observations suggested two different types of sequence associations between variants from various tissues, one of them appearing to be generated by the lymphoid system, which contained at least some of the isolates from each organ, the other was separate from a lymphoid origin, and closer to a neurotropic strain, which generally included colon variants and occasionally lung variants.

Three of the study subjects demonstrated a low viral load in the four examined organs. One of them, NA98028, who died of illicit drug overdose in the presymptomatic stage, had a high CD4 count close to death (CD4: 703) and appeared to show no abnormalities in these four examined tissues. The relatively normal histological appearances observed in this individual, fit well with the low level of provirus detected. Two other subjects, NA97017 and NA96272 who died of AIDS-related illnesses, had relatively low CD4 counts at death (16 and 126 respectively,

which ranked in the middle of symptomatic individuals), one received anti-retroviral therapy during the symptomatic stage (NA97017), the other was not treated. When compared with the other subjects, no significant differences were observed in the pathological conditions in these two subjects. The low levels of HIV-1 detected in these two individuals are difficult to explain, seemingly not due to the antiretroviral therapy, nor to the progression of disease or any other obvious cause. The possible reason might be due to inappropriate sampling. The organs examined in this study are made up of a large mass of solid tissue, where cellular movement is relatively restricted, unlike the peripheral circulation. It is therefore highly likely that separate populations could emerge within tissues following initial infection. Only a small amount of tissue is sampled for examination and it is possible that the infected areas were missed or that unrepresentative samples were selected. Similar problems were also observed in the brain tissue from subject NA97020, who had developed HIVE and showed p24 immunopositivity in brain, but PCR quantitation revealed only 6 copies per 10^6cells. It is unusual that cases with p24 immunopositivity but with low viral load by PCR quantitation in brain tissue should develop. HIVE. For further investigations, multiple sampling is clearly important.

5.4.3 Tissue-Specific Grouping

The HIV-1 population in an infected person often contains a diverse mix of variants. Populations of these variants infecting different tissues *in vivo* are generally distinct in the hypervariable V3 region of *env* gene (Ball *et al.,* 1994; Korber *et al.,* 1994). Several studies have identified distinct isolates from lung (Itescu *et al.,* 1994), colon (Barnett et al., 1991; van der Hoek et al., 1996) and brain (Donaldson *et al.,* 1994b; Power *et al.,* 1995; van't Wout *et al.,* 1998a; Gatanaga *et al.,* 1999; Morris *et al.* 1999), indicating compartmentalisation of HIV-1 in different tissues. In this study, variants recovered from lung, colon and brain demonstrated consistent sequence differences from those recovered from lymph node either in the V3 or $p17^{gag}$ regions. These tissue-specific groups provided evidence for specific cellular tropism, and most of these tissue-specific groupings occurred more often in the V3 than in the $p17^{gag}$ region. As described previously, genetic diversity in the V3 and $p17^{gag}$ regions is under different selection pressures. Being a major target for neutralising antibodies

(LaRosa *et al.,* 1990; Robert-Guroff *et al.,* 1994), the high degree of diversification in V3 regions is believed to be driven by a strong immune pressure, whereas in $p17^{gag}$, a bias for nonsynonymous substitutions suggests selection based on replication fitness (Kasper *et al.,* 1995). These observed tissue-specific groups might represent the differences in the rate of virus turnover under immune pressure in different tissues (van der Hoek *et al.,*1996; van der Hoek *et al.,*1998). They found that only one out of seven in their study group showing specific clustering in faecal sample (van der Hoek *et al.,*1998). In the present study, because comparison was made within various tissues, an even more complicated pattern was observed. In general, lung-specific clusters were observed within two subjects, colon-specific clusters were observed in three and brain-specific clusters were observed in two. These lineages frequently shared some sequences similarity with variants recovered from other tissues, although they were separated from LN variants. Most of the variants demonstrated a strong host-specific character rather than tissue-specific groupings (data of phylogenetic trees were not shown). For example, other analysis of phylogenetic trees prepared from all LN sequences, variants obtained from the same subject always clustered tightly, and separated from other subjects. Similar observations applied to lung, colon and brain. The heterogeneity between variants obtained within subject was less than variants obtained between subjects. Therefore, these tissue-distinct variants within individuals seemingly reflected the host immune selection, or time-related factors (van der Hoek *et al.,* 1998) rather than simple tissue tropism.

5.4.4 Genomic Recombination

Genomic recombination is one of the most important mechanisms by which new variants with different biologic and pathogenic properties can be produced. Also, it may play an important role in the evolution of drug-resistant strains and new envelope serotypes (Burke, 1997). It has been demonstrated earlier that superinfection of two defective viruses *in vitro* could produce cytopathic virus particles because of homologous recombination (Kishi *et al.,* 1995). Moreover, various types of recombinations, such as between different subtypes of HIV-1 group M (Gao *et al.,* 1998), between HIV_{LAI} and $HIV\text{-}1_{SF2}$ (Wei & Fultz, 1998), and even

between HIV-1 groups O and M (Takehisa *et al.,* 1998), were confirmed at a rather high frequency (Takehisa *et al.,* 1998). Most of the genetic recombination was observed occurring between *gag* and *env* regions (Carr *et al.,* 1996; Cornelissen *et al.,* 1996; Kampinga *et al.,* 1997).

A recent study demonstrated an extensive recombination between different regions of HIV-1 genome in brain infection (Morris *et al.,* 1999). In this study, variants infecting multiple regions of the brain of an individual with HIVE were assembled from two distinct $p17^{gag}$ lineages and a limited number of distinct hypervariable region lineages, often with different combinations within each histological regions of brain (Morris *et al.,* 1999). Similar to these observations, discordant phylogenetic relationships between $p17^{gag}$ and V3 region of HIV-1 genome were found in some of the present study subjects. For example, separate grouping of $p17^{gag}$ region sequences from lymph node and colon were observed in subject NA96425, whereas sequences in V3 from these tissues were interspersed. Similar findings in subject NA97021 showed that $p17^{gag}$ sequences from the brain were extremely diverse and polyphyletic, while V3 sequences clustered into two discrete clades. These observations support the previous findings that recombination could occur within an infected individual between variants descended in each case from the original infecting strain, and that different regions of HIV genome may follow different evolutionary selection. However, the small patient numbers and the limited genomic regions examined (only p17gag and V3) are potentially limiting to these results. For further investigation, multiple regions of the HIV genome in a greater number of cases should be analysed to further substantiate these findings.

Chapter 6: General Discussion

The new therapeutic strategy, highly active antiretroviral therapy (HAART) became available in the summer of 1996. Despite the fact that HAART has been confirmed as effective in decreasing AIDS-related morbidity and mortality, and in achieving sustained and progressive immunological reconstitution in both early and late HIV-1 infection (Li *et al.,* 1998; reviewed in Montaner *et al.,* 1998a), increasing numbers of reports have drawn attention to the severe long-term side effects of HAART (Carr *et al.,* 1998; Henry *et al.,* 1998), and the rapid rebound of viral replication occurring after cessation of drug therapy (Montaner *et al.,* 1998b; Chun *et al.,* 1999). According to the latest annual world health report, AIDS is now ranked at fourth place among all causes of death worldwide, and is the leading overall cause of death in Africa and developing countries (UNAIDS/WHO, 1999). Moreover, the HIV epidemic continues to spread at a rate of over 6000 new infections every day (UNAIDS/WHO, 1999). Responding to the challenge of this devastating pandemic, basic and clinical investigators in many regions of the world have rapidly advanced our understanding of the biology, immunology, and virology of systemic HIV-1 infection. A better understanding of HIV pathogenesis and the mechanisms underlying HIV-1 related disease in specific tissues is still a high priority in tracking the medical impact of the pandemic.

Infection with HIV-1 is associated with a slow, progressive and irreversible impairment of the immune system eventually leading to AIDS. Previous studies suggested that CD4+ lymphocytes were the major target for HIV-1 infection, and the dysfunction and depletion of CD4+ lymphocytes by HIV-1 was thought to be the major mechanism responsible for the progressive disease (Klatzmann *et al.,* 1984; Becherer *et al.,* 1990; Saksela *et al.,* 1994; Ho *et al.,* 1995). However, the recent discovery of secondary receptors (Bleul *et al.,* 1996; Deng *et al.,* 1996; Feng *et al.,* 1996; Liu *et al.,* 1996; Oberlin *et al.,* 1996; Paxton *et al.,* 1996; Samson *et al.,* 1996), and the demonstration of a wider range of host cells susceptible to HIV-1 *in vitro* (refer to chapter 4) suggested that the mechanisms involved in HIV-1 pathogenesis might be more complicated than previously thought. In the present study, immunostaining techniques together with nucleotide sequencing and phylogenetic analysis of viral isolates were employed in order to achieve a better understanding of

the mechanisms for viral dissemination throughout the body, and of the histologic and genetic characterisations of HIV-1 infection in the lymphoid and non-lymphoid organs.

Gathering all the information from the immunohistology and sequence analysis, the findings in this thesis document the complicated relationships between HIV-1 populations recovered from various organs within individuals, and suggest possible mechanisms of spread of the virus into non-lymphoid tissues.

According to the phylogenetic analysis, nucleotide sequences from the V3 and $p17^{gag}$ regions revealed a complex variety of relationships between variants recovered from different tissues, including lymph node (LN), lung, colon and left frontal lobe brain tissues. In general, at least some of the variants from each tissue were interspersed, and this observation suggested a shared population and target cell type between these tissues. However, the majority of variants recovered from the brain were found to be phylogenetically distinct from variants derived from LNs. Of the variants derived from the colon, sequences were sometimes interspersed with lymph node and lung isolates, but colon-specific variants were observed in two subjects (NA97021 and NA98025). These colon-specific isolates were phylogenically distinct from LN lineages but demonstrated certain sequence similarities with brain variants. Most of the variants from the lung were interspersed with those from LN, but in one case (NA98025), lung variants were clustered with colon isolates and phylogenetically distinct from LN variants.

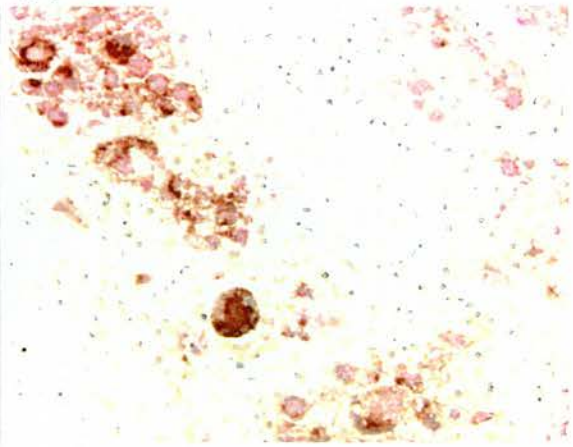
In contrast to these complex phylogenetic relationships, the pattern of immunopositivity for HIV-1 p24 antigen was relatively simple. Most LN sections obtained either from pre-symptomatic or from symptomatic patients displayed p24 immunopositivity within follicular dendritic cells. In brain tissues, the majority of p24 positive cells were multinucleated giant cells (MGCs), microglial cells and macrophages, and the presence of p24 immunopositivity were largely associated neuropathological abnormalities, most often with a tissue damaging form of HIV encephalitis. Other tissues, including lung and gastrointestinal tract were usually p24

negative, and only in some symptomatic subjects, p24 signal was detected within secondary lymphoid follicles in these organs.

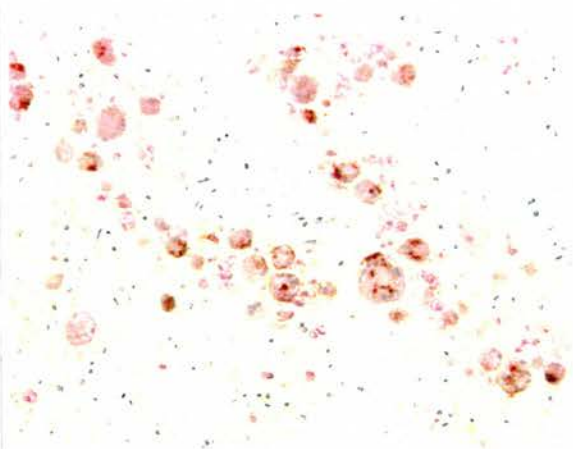
These observations suggested at least two mechanisms for spread of HIV-1 into non-lymphoid tissues. For tissues such as the lung and the gastrointestinal tract, combined immunohistochemical and genetic observations suggest that infection of these tissues arose directly from infiltration by lymphocytes. A different mechanism seems likely for HIV-1 variants detected in the brain, which were found predominantly in cells of macrophage lineage, and these formed genetically distinct populations from those in lymphoid tissues. Moreover, the colon and lung variants from subjects NA97021 and NA98025, which were distinct from LN lineages but similar in sequences with brain lineages provide evidence suggesting that infection of lung and colon may also occur through macrophage involvement, although there was no immunohistological evidence for productively infected macrophages in lung and colon in these individuals.

These finding confirm and extend the finding of previous studies (Donaldson *et al.,* 1994b), when the V3 region was sequenced from various organs, including brain, colon, lung and LN from HIV-infected individuals. In one particular case, NA91246 (P4), frequent p24 antigen-expressing lung macrophages were found and these infected cells formed pronounced multinucleated syncytia (MGCs), similar to the pattern of brain infection. The predominant V3 sequence of provirus amplified from lung tissue was identical to the major variants infecting the brain and to a proportion of those in colon tissue, but distinct from those derived from LN. Interestingly, in another subject NA92118 (P5), immunostaining demonstrated p24 positivity within the lymphoid infiltration but not macrophages in lung, and sequences from V3 region were interspersed with LN isolates but not brain isolates (Figure 6. 1 & Figure 6. 2).

(a)



(b)



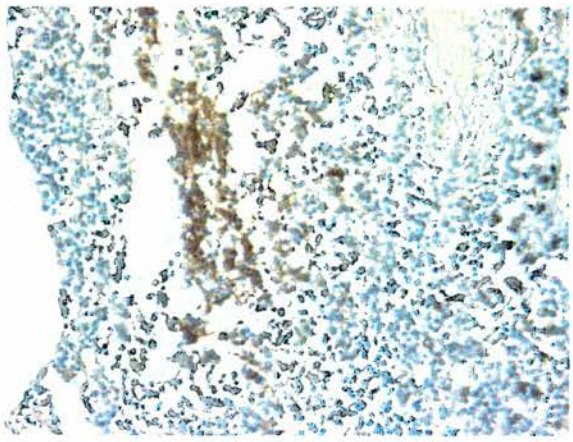
(c)

Consensus	CTRPNNNTRK SIHIGPGRAF YTTGEIIGDI RQAHC	n
Brain	....L...S.. .A..D.....	12
	....L...S.. ....D.....	2
	....S.. .A..D.....	2
	....N..... ....D.....	1
Spinal cord	....L...S.. .A..D.....	6
	....S.. .A..D.....	1
Colon	....S..... ..N.....I ....D.....	6
	....L...S.. .A..D.....	5
	....S..... ....D.....	1
	....L...S.. ....D.....	1
	....I ....D.....	1
	....S.. ....D.....	1
	....L...S.I .A..D.....	1
Lung	....L...S.. .A..D.....	7
	....S..... G.....	3
	....S..... ....S.. ....D.....	1
	....A..S.. .A..D.....	1
	....N..... ....D.....	1
	....S..... ..N..... ....D.....	1
	....L...S.. ....D.....	1
PBNCS	....S..... G.....	12
	....S..... ..N..... ....D.....	1
	....S..... G..... ....D.....	1
Spleen	....S..... G..... ....D.....	1
	....D.....	1
Lymph node	....S..... G.....	5
	....S..... ..N..... ....D.....	3
	....S.. .A..D.....	2
	....	2
	....S..... G..... ....D.....	1
	....L...S.. .A..D.....	1
	....N..... ....D.....	1

Figure 6. 1. Morphology and double immuno-labelling detection of p24 antigen and macrophage cell marker in the lung section from subject NA91246 demonstrating a high level macrophage syncytia (p24: DAB, PGM1: Vecter Red, with haematoxylin counter stain. Magnification: (a) x200, (b) x200). In this case, the predominant V3 sequence of provirus amplified from lung tissue was identical to the major variants infecting the brain (c).

(a)

(b)



(c)

Consensus	CTRPNNNTRK SIHIGPGRAF YTTGEIIGDI RQAHC	n
Brain	.... .A.....	15
	...L..... ..... .A.....	2
Lung	.... .A..D.....	10
	....P..... .A..D.....	3
PBNCS	.... .A..D.....	13
	.....K.... ..... .A..D.....	2
	....P..... .A..D.....	1
Lymph node	.... .A..D.....	12
	....P..... .A..D.....	1

Figure 6. 2. HIV-1 p24 detection in the lung section taken from subject NA92118 demonstrating a high level of lymphocytic infiltration (DAB with haematoxylin counter stain. Magnification: x200). In this case, the predominant V3 sequence of provirus amplified from lung tissue was identical to the variants isolated from PBMCs and LN, but distinct to the brain variants.

The finding that p24 positivity observed in lung MGC/macrophages showed a close V3 phylogenetic relationship with brain isolates, whereas p24 positivity observed in lung lymphoid infiltration showed a mixed LN/lung phylogenetic pattern, correlated closely with the findings of the present study. Although p24 immunostaining failed to identify the infected cell type in lung and colon, these similar V3 sequences observed between lung, colon and brain isolates suggests that infection in these instances occurred through a transmission mechanism might be the same in these three tissues.

Productive replication of HIV-1 in brain MGCs, macrophages and microglia is a critical component of viral neuropathogenesis (Bell, 1998; Ghorpade *et al.,* 1998). However, the formation of p24-positive-MGC is a rare event outside the CNS, and p24-positive-macrophages were found only occasionally in the present study. In a recent case report, this characteristic cell type was identified in hyperplastic gut-associated lymphoid tissue of an HIV-infected patient (Lewin-Smith *et al.,* 1999). In this case, HIV-1 RNA expression and p24 positivity was demonstrated in MGCs, macrophages and FDCs using in situ hybridisation and immunostaining. Unfortunately, no sequence data was available for this patient (Lewin-Smith *et al.,* 1999), which might have helped to prove the macrophage-lineage hypothesis described above. From the V3 region sequences, apparently these neurotropic-similar HIV-1 isolates have commonly contained neutral amino acids at position 11 and acidic residues at position 25, and were predicted as CCR5-dependent (NSI) strains. CCR5-dependent strains have been found predominantly in acutely infected individuals, and are most transmissible (Zhu *et al.,* 1993; Zhu *et al.,* 1995; Spira *et al.,* 1996). It has been postulated that these CCR5 dependent strains are the principal virus type responsible for person-to-person transmission and viral persistence early in infection (Collman & Yi, 1999).

A recent study cloning brain-derived and colon-derived viruses into an isogenic (NL4-3) viral background has reported that CCR5 is a primary coreceptor for these brain- and colon-derived viruses, and suggested that tissue infection may not depend significantly on changes in coreceptor usage, but rather selects for CCR5 use

throughout disease progression (Chan *et al.,* 1999). In this regard, cells of the macrophage lineage, such as microglia and tissue macrophages which present both CCR5 and CD4 molecule on their cell surface, are possibly the principal cells that establish and maintain virus populations in all tissues.

Several studies have demonstrated that the first targets of HIV-1 are cells of the macrophage lineage, especially in mucosal areas, such as lung and colon (van't Wout *et al.,* 1994; Donovan *et al.,* 1996; Goletti *et al.,* 1996; Marx *et al.,* 1996; Spira *et al.,* 1996; Kahn & Walker, 1998; Hirsch *et al.,* 1998). Although Zhang *et al.* (1999) demonstrated that soon after initial infection, macrophages can pass infection to CD4+ T cells in cell-cell interactions that activate the T cells (Zhang *et al.,* 1999), these productively infected CD4+ T cells are later eliminated by the host immune system (reviewed in Levy, 1998). As disease progresses, consistent loss of CD4+ T cells leads to immunodeficiency and increasing susceptibility to a broad range of opportunistic pathogens, and infections with opportunistic pathogens are usually accompanied by mostly reversible increases in HIV-1 viremia (Ghassemi *et al.,* 1995; Goletti *et al.,* 1996; also reviewed in Levy, 1998). A recent study by Orenstein *et al.* demonstrated that while CD4+ lymphocytes decline dramatically in the later stages of HIV-1 infection, macrophages were identified as highly productive sources of HIV-1 (Orenstein *et al.,* 1997a). Thus, with reference to the findings of this thesis, the brain-derived, colon-derived and lung-derived CCR5-dependent isolates were quite possibly derived from a common cellular source. Moreover, these isolates that were distinct from those derived from lymphoid lineage were found coincidentally within subjects who had developed systemic pathological abnormalities, including HIVE (NA97021 & NA98025 in the present study and P4 (NA91246) in Donaldson *et al.,* 1994b). This observation suggests that this CCR5- or macrophage-dependent lineage represent a disease-inducing lineage, which is responsible for the systemic spread of infection throughout the body. As for the observed tissue-tropic property, it might merely reflect differences in the rate of virus turnover in different cell types under different selective pressures (van der Hoek *et al.,* 1998; Morris *et al.,* 1999. Also refer to section 5.4.4).

Although expressing much lower levels of cell surface CD4 molecules than helper T lymphocytes, cells of the macrophage lineage could be infected through a CCR5-dependent mechanism (Berger, 1997; Berger *et al.,* 1999), or through an efficient gp120/gp41-independent route, such as phagocytosis of other infected cells or by Fc receptor-mediated endocytosis of antibody-coated HIV virions, since the HIV-1 virion may be found sequestered in intracellular vacuoles (Gendelman *et al.,* 1989; Abbas *et al.,* 1999). This mechanism has also been proposed for the latent infection of astrocytes which may complicate productive HIVE in some cases (Hao & Lyman, 1999). Infection of macrophage lineage cells is associated with low-level production of virus, frequent latent infection, and relative resistance to the cytopathic effects of HIV-1 (Stent *et al.,* 1997; Abbas *et al.,* 1999; reviewed in Levy, 1998). A recent study demonstrated that protease inhibitors were less active in chronically infected macrophages than in chronically infected lymphocytes, and when protease inhibitors were removed, production of virus rapidly returned to the levels found in untreated cells (Perno *et al.,* 1998). This suggested that variants infecting macrophages may be more resistant to antiviral treatment. These species, together with follicular dendritic cell-associated viruses (refer to section 4.3.1), have been thought to be responsible for the rapid rebound viremia after cessation of HAART (Perno *et al.,* 1998; Orenstein *et al.,* 1999b), and form the most serious challenge to HIV therapy.

As described above, most macrophages appear to be infected latently, except in the CNS. This may be the reason that macrophages were found to be p24 positive only very rarely in the immunohistochemistry trial. For further evidence supporting this supposition that macrophage-lineage and subspecies are the major cause of systemic spread, it is important to identify the infected cells, whether productively or latently infected. Clearly, immunostaining to identify viral protein was insufficient, even the sensitivity has been significantly improved (refer to chapter 3). For further understanding of HIV pathogenesis, reconciliation of both clinical and pathological observations with virological studies is indispensable. In the future, *in-situ* PCR based on HIV proviral sequences combined with immunostaining for cell surface markers might be employed to investigate the cell types which are latently or non-

productively infected and therefore provide a more complete description of the cellular targets of HIV-1 *in vivo*. Phenotypic characterisation of HIV-1 variants infecting different cell types would also be of value in understanding the mechanism of the observed genetic compartmentalisation between tissues, and the extent to which sequence differences represent adaptation for replication in different cell types. Also, for the large organs, such as brain, lung and gastrointestinal tract, multiple sampling is required for more accurate characterisation of the viral species involved.

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APPENDIX I. Initials for DNA, RNA and Amino acid.

(A). DNA and RNA Initial

Symbols	Name	Remarks
A	Adenine	Purine
G	Guanine	Purine
C	Cytosine	Pyrimidine
T	Thymine	Pyrimidine
U	Uracil	Pyrimidine

(B). Amino Acid Initial

Symbols	Name	Remarks
A	Alanine	Ala
C	Cysteine	Cys
D	Aspartic acid	Asp
E	Glutamic acid	Glu
F	Phenylalanine	Phe
G	Glycine	Gly
H	Histidine	His
I	Isoleucine	Ile
K	Lysine	Lys
L	Leucine	Leu
M	Methionine	Met
N	Asparagine	Asn
P	Proline	Pro
Q	Glutamine	Gln
R	Arginine	Arg
S	Serine	Ser
T	Threonine	Thr
V	Valine	Val
W	Tryptophan	Trp
Y	Tyrosine	Tyr

Key:

[a]: Risk Groups: **PDM** --- pre-AIDS Drug Misuser; **ADM** --- AIDS Drug Misuser
P-Hetero --- pre-AIDS heterosexual; **AH** --- AIDS Homosexual

[b]: CD4/CD8: The ratio of last CD4 counts and CD8 counts

[c]: **Rx**: drug therapy

[d]: **MAI**: *Mycobacteria avium-intracellulare*

[e]: ***Macro*:** Macroscopy examination

[f]: ***Micro*:** Microscopical examination

[g]: **OI:** Opportunistic Infection

[h]: **N:** Necrosis

[i]: **IHC results**: **0**: p24 negative; **1**: p24 weakly positive; **2**: p24 intermediate positivity; **3**: p24 strongly positive. The number of tissue blocks examined from each organ is indicated in brackets.

[j]: **PCP:** *Pneumocystis carinii* pneumonia

[k]: **KS:** Kaposi's Sarcoma

APPENDIX II. General information and pathological findings for study subjects

No.	NA97021	NA97020
Age/Gender	49 / F	49 / M
Risk Group[a]	ADM	AH
CD4 / CD8[b]	137 / 1620	8 / 388 [f]
Date of first HIV+ test	**18/Nov/1984**	**01/Jan/1991**
Rx[c] **during Pre-AIDS**	25/Sep/90 ~ 11/Jun/91 --- AZT 11/Jun/91 ~ 24/Sep/91 --- No Rx 24/Sep/91 ~ 23/Jan/92 --- AZT 23/Jan/92 ~ 01/Jan/93 --- No Rx	No Rx
Date of AIDS	**01/Jan/1993**	**01/Jun/1994**
AIDS Presenting Illness	HIV Encephalopathy	MAI[d]
Rx during AIDS	01/Jan/93 ~ 09/Jan/96 -- No Rx 09/Jan/96 ~ 27/Feb/96 -- AZT+ddC 27/Feb/96 ~ 27/Jan/97 --- No Rx	17/Dec/96 ~ 27/Jan/97 --- d4T
Date of Death	**28/Jan/1997**	**28/Jan/1997**
Clinical Summary	1. Drug and alcohol use 2. Hepatitis C positive 3. Recurrent oesophageal candidiasis 4. Chest infections 5. Dementia 6. Parkinsonian symptoms 7. AIDS	1. Two weeks history of persistent diarrhoea (due to *Clostridium difficile* which was treated) 2. Past history of severe weight loss, low white cell and platelet counts 3. Infection with atypical mycobacteria (MAI) 4. Urinary tract infection 5. Bronchopneumonia 6. AIDS
Interim Findings at autopsy	• Terminal chest infection in the context of severe immune depression due to AIDS. • ? HIV Encephalopathy.	• Bronchopneumonia. • Cachexia. • AIDS.
Final autopsy diagnosis	1. Bronchopneumonia 2. HIV encephalitis 3. Hepatic fibrosis 4. AIDS	1. Bronchopneumonia 2. HIV encephalitis 3. Hepatic steatosis 4. AIDS

APPENDIX II. General information and pathological findings for study subjects (continued)

No.	NA97021 (continued)	NA97020 (continued)
Pathological Findings in **Spleen**	*Macro*[e] --- Weight 187g, appeared reactive. *Micro*[f] --- Slight lymphocyte depletion. No OI[g] or N[h] *IHC*[i] --- p24:3+ (x1)	*Macro* --- Weight 130g, appeared congested. *Micro* --- Moderate lymphocyte depletion. No OI or N *IHC* --- p24:0 (x2)
Pathological Findings in **Lymph Nodes**	*Macro* --- Appeared reactive and largest in the para-aortic region. *Micro* --- Slight lymphocyte depletion No OI or N *IHC* --- p24:3+ (x1)	*Macro* --- Appeared reactive *Micro* --- Lymphocyte depletion No OI or N *IHC* --- p24:0 (x1)
Pathological Findings in **Lung**	*Macro* --- RL 392g, LL 386g. Right lower lobe was consolidated and showed pleural adhesions *Micro* --- Acute bronchitis, focal bronchopneumonia with aspiration. No OI or N *IHC* --- p24:2+ (x1); 0 (x1)	*Macro* --- RL 782g, LL 695g. *Micro* --- Severe bronchopneumonia with foreign body giant cells. No OI or N *IHC* ---p24: 0 (x3)
Pathological Findings in **Intestine**	*Macro* --- Normal *Micro* --- No abnormalities within limits of slight autolysis No OI or N *IHC* --- p24: Ile 0 (x1); Colon 0 (x1)	*Macro* --- Normal *Micro* --- No abnormalities within limits of slight autolysis No OI or N *IHC* --- p24: Ile 0 (x2); Colon: 0 (x2)
Pathological Findings in **Brain**	*Macro* --- Fixed weight 985g. Atrophy, no focal lesions *Micro* --- HIVE with unusual degree of lymphocytic infiltration. No OI or N *IHC* --- p24: 3+ (x1)	*Macro* --- Fixed weight 1471g. Slight atrophy, no focal lesions *Micro* --- HIVE with neurodegenerative changes (Beta amyloid precursor protein and ubiquitin present in white matter) No OI or N *IHC* --- p24: 1+ (x1)

APPENDIX II. General information and pathological findings for study subjects (continued).

No.	NA97017	NA96371
Age/Gender	32 / M	34 / M
Risk Group[a]	ADM	AH
CD4 / CD8[b]	16 / 120	3 / 100
Date of first HIV+ test	**28/Oct/1985**	**12/Aug/1992**
Rx[d] during Pre-AIDS	No Rx	No Rx
Date of AIDS[e]	**01/Jun/1995**	**May/1994**
AIDS Presenting Illness	PCP[j]	KS[k]
Rx during AIDS	16/Apr/96 ~ 24/Dec/96 - AZT + ddC 24/Dec/96 ~ 24/Jan/97 - 3TC + d4T	AZT until close to death (no details)
Date of Death	**25/Jan/1997**	**01/Nov/1996**
Clinical Summary	1. Maintained on methadone but has been anaemic and pancytopenic 2. Recurrent chest infections 3. Pneumocystis prophylaxis 4. PCP 5. AIDS	1. Kaposi sarcoma 2. Chest infections and very troublesome peripheral oedema due to a combination of anaemia and hypoalbuminaemia 3. Consistently negative for PCP
Interim Findings at autopsy	• Florid pulmonary oedema accompanied by serous effusions in all the body cavities. • ? PCP. • Cardiomyopathy. • AIDS.	• Renal and hepatic failure due to malignant compression (KS) of liver and kidney • AIDS
Final autopsy diagnosis	1. Pulmonary oedema and haemorrhage 2. Serous cavity effusions 3. Cardiomyopathy 4. AIDS	1. Extensive cutaneous, pulmonary, retroperitoneal and pelvic KS which also involves the bladder wall, liver and LNs 2. CMV infection of adrenal gland and cerebrum 3. AIDS

APPENDIX II. General information and pathological findings for study subjects (continued)

No.	NA97017 (continued)	NA96371 (continued)
Pathological Findings in **Spleen**	*Macro* --- Weight 330g, appeared congested. *Micro* --- Lymphocyte depletion. No OI or N *IHC* --- p24:0 (x1)	*Macro* --- Weight 235g, congested *Micro* --- Lymphocyte depletion No OI or N *IHC* --- p24:3+ (x1)
Pathological Findings in **Lymph Nodes**	*Macro* --- Appeared inconspicuous *Micro* --- Lymphocyte depletion No OI or N *IHC* --- p24:0 (x1)	*Macro* --- Many LNs infiltrated by KS *Micro* --- KS *IHC* --- p24:1+ (x1)
Pathological Findings in **Lung**	*Macro* --- RL 950, LL 800. Oedema and haemorrhage *Micro* --- Gross oedema and focal emphysema with pigmented macrophages No OI or N *IHC* --- p24:0 (x3)	*Macro* --- RL 621g, LL 654g. RL compressed by the large right pleural effusion, LL haemorrhage and bronchopenumonia *Micro* --- KS, Haemorrhage No OI *IHC* --- p24:0 (x2)
Pathological Findings in **Intestine**	*Macro* --- Normal *Micro* --- No abnormalities within limits of slight autolysis No OI or N *IHC* --- p24: Ile:0 (x1); Colon:0 (x2)	*Macro* --- Oedema, otherwise normal *Micro* --- No other abnormalities within limits of autolysis No OI or N *IHC* --- p24: Ile:0 (x1); Colon:0 (x2)
Pathological Findings in **Brain**	*Macro* --- Fixed weight 1392g, Mild atrophy, no focal lesions *Micro* --- Slight Alzheimer type II astrocytosis. No HIVE, OI or N *IHC* --- p24: 0 (x1)	*Macro* --- Fixed weight 1387g, No atrophy or focal lesions *Micro* ---CMV micronodular encephalitis No HIVE, OI or N *IHC* --- p24:0 (x1)

APPENDIX II. General information and pathological findings for study subjects (continued)

No.	NA96425	NA96272
Age/Gender	32 / M	32 / M
Risk Group[a]	ADM	ADM
CD4 / CD8[b]	1 / 298	126 / 636
Date of first HIV+ test	**05/Apr/1984**	**26/May/1987**
Rx[d] during Pre-AIDS	No Rx	No Rx
Date of AIDS[e]	No AIDS-related illness (AIDS was diagnosed on the CD4 count alone)	No AIDS-related illness (AIDS was diagnosed on the CD4 count alone)
AIDS Presenting Illness	N/A	N/A
Rx during AIDS	No Rx	No Rx
Date of Death	**11/Dec/1996**	**13/Aug1996**
Clinical Summary	1. Heavy alcohol user in the past 2. Drug habit is maintained on Dihydrocodine and Temazepam 3. Chest infections 4. CD4 zero for more than one year 5. CMV antibody positive 6. With jaundice and mild confusion but apyrexial 7. Had diarrhoea and chest signs and developed low output cardiac failure?cardiomyopathy and probable sepsis	1. Ten years HIV with hepatitis B &C positive 2. Episode of abdominal pain 3. Emergency complaining of chest pain and a right pleural effusion was discovered
Interim Findings at autopsy	• Bronchopneumonia, heart and liver failure • Cardiomyopathy • Cirrhosis • AIDS (on CD4 count alone)	• Bronchopneumonia • Cirrhosis • AIDS (on CD4 count alone)
Final autopsy diagnosis	1. Bronchopneumonia 2. Hypertrophic cardiomyopahty 3. Cirrhosis 4. Hepatic encephalopathy 5. Old cerebellar infarct 6. AIDS	1. Bronchopneumonia and right pleurisy 2. Jaundice & cirrhosis 3. Portal hypertension, ascites and oesophageal and anal varices 4. Splenomegaly 5. Hepatic encephalopathy 6. AIDS

APPENDIX II. General information and pathological findings for study subjects (continued)

No.	NA96425 (continued)	NA96272 (continued)
Pathological Findings in **Spleen**	*Macro* --- Weight 672g, very congested *Micro* --- Congested in keeping with cirrhosis. Lymphocyte depletion No OI or N *IHC* --- p24:3+ (x1)	*Macro* --- Weight 619g, very congested *Micro* --- Grossly congested in keeping with cirrhosis No OI or N *IHC* --- p24:3+ (x2)
Pathological Findings in **Lymph Nodes**	*Macro* --- Lymphadenopathy *Micro* --- Sinus hyperplasia No OI or N *IHC* --- p24:1+ (x1)	*Macro* ---Normal *Micro* --- Germinal follicles present in some LNs No OI or N *IHC* --- p24:1+ (x1)
Pathological Findings in **Lung**	*Macro* ---RL 1065g, LL 1207g Bronchopneumonia *Micro* --- Bronchopneumonia No OI or N *IHC* --- p24: 1+ (x1); 0 (x2)	*Macro* --- RL 1209g, LL 818g. Congestion, haemorrhage and right bronchopneumonia *Micro* --- Severe oedema, numerous pigment macrophages, right bronchopneumonia No OI or N *IHC* --- p24: 0 (x2)
Pathological Findings in **Intestine**	*Macro* ---Oedema, otherwise normal *Micro* --- No other abnormalities within limits of autolysis No OI or N *IHC* --- p24; Ile:0 (x1); Colon: 0 (x1)	*Macro* --- Oedema, melaena, otherwise normal *Micro* --- Sparse acute inflammatory infiltrate is noted in the oedematous serosa of the bowel No OI or N *IHC* --- p24; Ile:0 (x1); Colon:2+ (x1)
Pathological Findings in **Brain**	*Macro* --- Fixed weight 1476g, No atrophy, old right cerebellar infarct *Micro* --- Old cerebellar infarct confirmed No HIVE, OI or N *IHC* --- p24: 0 (x1)	*Macro* --- Fixed weight 1701g, No atrophy or focal lesions *Micro* --- No HIVE, OI or N *IHC* --- p24: 0 (x1)

APPENDIX II. General information and pathological findings for study subjects (continued)

No.	NA98025	NA98028
Age/Gender	43 / M	48 / F
Risk Group[a]	P-Hetero	PDM
CD4 / CD8[b]	297 / 232	703 / 4091
Date of first HIV+ test	**01/Jan/1992**	**07/Apr/1984**
Rx[d] **during Pre-AIDS**	No Rx	No Rx
AIDS Presenting Illness	Clinically in **Pre-AIDS**	Clinically in **Pre-AIDS**
Date of Death	**20/Jan/1998**	**27/Jan/1998**
Rx during AIDS	1. heavy drinking and poor diet 2. shortness of breath and pain in the chest and was found to have pneumonia, went into respiratory failure 3. Renal failure	1. IVDU 2. Diazepam: 15 mg/nightly Cetrizine: 10 mg/nightly Amitryptaline: 50 mg/nightly Trazodene: 100 mg/nightly Codanthrusate: 2 capsules/nightly Stemetil – 5mg 3 times a day Vitamins B12 complex strong
Clinical Summary	• Bronchopneumonia, cirrhosis, and acute renal failure. • HIV positive at Pre-AIDS stage.	• Sudden death by illicit drug overdose. • Old hemiplegia. • HIV positive at Pre-AIDS stage.
Final Autopsy Diagnosis	1. Bronchopneumonia 2. Hepatic cirrhosis and necrosis 3. Renal tubular necrosis 4. Pancreatic fibrosis 5. HIV positive man with probable incipient HIV encephalitis	1. Extensive old right cerebral infarct 2. No evidence of HIV encephalitis 3. HIV positive woman

APPENDIX II. General information and pathological findings for study subjects (continued)

No.	NA98025 (continued)	NA98028 (continued)
Pathological Findings in **Spleen**	*Macro* --- Weight 260g, septicaemia appearance *Micro* --- No lymphocyte depletion, No OI or N *IHC* --- p24:3+ (x1); 1+ (x1)	*Macro* --- Weight 195g, appeared normal *Micro* --- Not available *IHC* --- p24:0 (x1)
Pathological Findings in **Lymph Nodes**	*Macro* --- Reactive lymphadenopathy *Micro* --- Lymphoid follicles are present with sinus hyperplasia No OI or N *IHC* --- p24:3+ (x8); 1+ (x1); 0 (x1)	*Macro* --- Reactive lympadenopathy *Micro* --- Acute lymphadenitis No OI or N *IHC* --- p24:3+ (x4); 1+ (x1)
Pathological Findings in **Lung**	*Macro* --- RL 2406g, LL 1670g. Consolidation *Micro* --- Bronchopneumonia and haemorrhage No OI or N *IHC* --- p24:1+ (x1); 0 (x1)	*Macro* --- RL 570g, LL 460g Appeared normal *Micro* --- Oedema, No OI or N *IHC* --- p24:0 (x2)
Pathological Findings in **Intestine**	*Macro* --- Oedema, melaena, otherwise normal *Micro* --- No other abnormalities within limits of autolysis No OI or N *IHC* --- p24 Int :2+ (x2); 0 (x5)	*Macro* --- Normal *Micro* --- Persisting mucosal lymphoid follicles within limits of autolysis No OI or N *IHC* --- p24:0 (x4)
Pathological Findings in **Brain**	*Macro* --- Fixed weight 1393g No atrophy or focal lesions *Micro* --- Widespread but subtle microglial infiltrate in the white matter; lymphocytic infiltrate is noted; one giant cell is identified *IHC* --- p24:0 (x1)	*Macro* --- Fixed weight 802g Large right cerebral infarct *Micro* --- Old cerebral infarct No HIVE, OI or N *IHC* --- p24:0 (x1)

APPENDIX II. General information and pathological findings for study subjects (continued)

No.	NA97097
Age/Gender	36 / M
Risk Group[a]	PDM
CD4 / CD8[b]	Not available (not regular medical care)
Date of first HIV+ test	reportedly **April/1986**
Rx[d] **during Pre-AIDS**	No information
AIDS Presenting Illness	Clinically in **Pre-AIDS**
Date of Death	**08/Apr/1997**
Clinical Summary	1. User of street drugs 2. Chronic alcohol use 3. No Medical care 4. Reportedly (family) HIV positive in April 1986
Clinical cause of Death	• Suspected illicit drug and alcohol overdose. • HIV positive at Pre-AIDS stage.
Final Autopsy Diagnosis	1. Cerebral oedema 2. Slight focal chronic leptomeningitis 3. No evidence of HIV encephalitis 4. HIV positive man
Pathological Findings in **Spleen**	*Macro* --- Weight 280g, normal *Micro* --- Congested. No OI or N *IHC* --- p24:2+ (x1)
Pathological Findings in **Lymph Nodes**	*Macro* --- Normal *Micro* --- Sinus herplasia. No OI or N *IHC* --- p24:3+ (x1)
Pathological Findings in **Lung**	*Macro* --- RL 1190g, LL 790g. Right bronchopneumonia *Micro* --- Bronchopneumonia. No OI or N *IHC* --- p24:0 (x2)
Pathological Findings in **Intestine**	*Macro* --- Normal *Micro* --- No abnormalities within limits of autolysis. No OI or N *IHC* --- p24: NA
Pathological Findings in **Brain**	*Macro* --- Fixed weight 1303g; No atrophy or focal lesions *Micro* --- Slight lymphocytic infiltrate of leptomeninges. No HIVE, OI or N *IHC* --- p24: 1+ (only in lymphocytic infiltrate of leptomeninges, and within CD8 positive cell group)